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Mechanisms of Protective Activity of West Nile Virus Anti-Envelope Antibodies In vitro and In vivo

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ABSTRACT OF THE DISSERTATION

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In vitro and In vivo

by

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West Nile virus (WNV) is a neurotropic flavivirus capable of causing severe disease and death in humans. Studies in mice have demonstrated that the humoral immune response against WNV limits primary infection and protects against a secondary challenge. Accordingly, passive transfer of immune serum or monoclonal antibodies (MAb) against the envelope (E) protein either prior to WNV infection or shortly thereafter is sufficient to protect mice from disease. The E protein is an immunodominant antigen in the antibody response to WNV infection, and the most potent neutralizing MAbs recognize an epitope on the lateral ridge of domain III (DIII-LR) of the E protein. However, studies with serum from human patients show that antibodies against the DIII-LR epitope comprise at best, a minor component of the human anti-WNV antibody response. Rather, the human anti-E protein response is more dominantly directed against an epitope on the fusion loop of domain II (DII-FL), and antibodies against this epitope neutralize infection poorly in vitro. The studies described in this thesis examined how the antibody response to WNV is protective despite being skewed away from the most

potently neutralizing epitope (DIII-LR). In the first section, two WNV-specific human MAbs that were isolated from B cell populations of convalescent patients that strongly neutralized WNV infection in vitro and protected mice against lethal infection in vivo were characterized in detail. The MAbs were localized to E protein epitopes that are present only on intact WNV virions and subviral particles, and neutralization of infection occurred through a viral fusion blockade mechanism, similar to DIII-LR MAbs. This suggests that WNV infection may indeed induce strongly neutralizing antibodies, which simply go undetected by current epitope-specific diagnostics that utilize recombinant, monomeric E protein. Subsequently, a DII-FL mouse MAb that was non-neutralizing in vitro was shown to protect mice from WNV infection in vivo via its Fc effector functions, requiring C1q, Fc-gamma receptor III (CD16), and phagocytic cells. This highlights the limitations of current in vitro surrogate markers of protection, which cannot account for the beneficial effects conferred by immunodominant DII-FL antibodies. Overall, these studies provide an enhanced understanding of the mechanisms of protection of the human anti-WNV antibody response.

Chapter I

Introduction

West Nile virus disease. West Nile virus (WNV) is a mosquito-borne flavivirus that is capable of infecting humans and causing severe disease and even death. WNV belongs to the Flavivirus genus within the *Flaviviridae* family. Other members of this genus that cause significant human disease include St. Louis encephalitis (SLEV) and Japanese encephalitis (JEV) viruses, which are grouped into the JEV serocomplex along with WNV, as well as dengue (DENV), yellow fever (YFV), and tick-borne encephalitis (TBEV) viruses (reviewed in (55, 56)).

WNV cycles in nature between several species of birds and mosquitoes, with humans and other mammals as dead-end hosts (36, 77). Most human infections are asymptomatic, but ~20% result in a mild febrile illness. Less than one percent of infections cause severe neuroinvasive disease, which can produce neurologic sequelae lasting for months to years, and even death (24, 88, 89). Risk factors for symptomatic infection and disease include an age of greater than 55 years, a compromised immune status, genetic variation in the OAS1 gene, and a CC5Δ32 genotype (25, 32, 53, 54). There is currently no approved vaccine or therapy for WNV infection, although both are being actively developed (4).

WNV first appeared in the Western hemisphere in 1999 in New York and spread rapidly through North America, with thousands of severe human cases annually in the United States. Curiously, though, few human cases have been reported in Latin America despite the migration of avian hosts and appropriate vectors for transmission (47, 76). WNV also cycles endemically in the "Old World" of Central Europe, the Middle East, and Northern Africa (83).

Flavivirus infection. The flavivirus life cycle begins with binding to poorly characterized cell surface receptors. Upon binding, internalization of flaviviruses is believed to occur through receptor-mediated, clathrin-dependent endocytosis (1, 101, 102). After trafficking to $Rab5^+$ and/or $Rab7^+$ endosomes (48, 101), the mildly acidic pH within the lumen induces structural alterations in the flavivirus E protein (13, 62), which includes changes in its oligomeric state (13, 62, 92). During this process, also known as type II fusion, the hydrophobic peptide on the fusion loop of domain II of the E protein inserts into the endosomal membrane, thus physically joining the host and viral membranes, which allows the infectious RNA genome to enter the cytoplasm (44, 45).

Flaviviruses have a ~11 kilobase, positive polarity, single-stranded RNA genome. Upon release into the cytoplasm, the genome is translated as a single polypeptide, which is then cleaved by host and viral proteases into three structural (capsid [C], pre-membrane [prM], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (reviewed in (55, 56)). The production of nonstructural proteins allows for creation of negative sense RNA copies of the genome, each of which can serve as a template for the replication of multiple copies of positive sense genomes. These nascent genomes can either serve as template for translation or be packaged into progeny virions (15).

After translation of input strand RNA and viral replication, progeny virion assembly occurs within the endoplasmic reticulum (ER), with the capsid protein and genomic RNA in the cytoplasm associating with prM and E proteins in the lumen (55). Virus particles bud into the lumen of ER as immature virions (106). Transit of the immature virion through the mildly acidic compartments of the trans-Golgi network

(TGN) increases the susceptibility of prM to cleavage by a furin-like serine protease (52, 104). Release of prM is required for optimal infectivity (38) and completes the maturation process, occurring upon secretion of the virion into the neutral pH of the extracellular space (104). While cleavage of prM is a required step in the viral lifecycle, it can be an inefficient process in some cell types. Moreover, partially mature flavivirus virions containing some uncleaved prM also retain infectivity (37, 68). Adding even further complexity, the E protein rearrangements of maturation can nucleate from more than one point, creating multiple structural protein environments within a single virion (80).

E protein structure. X-ray crystallography studies have elucidated the three-domain structure of the flavivirus E protein (40, 61, 63, 72, 84, 108). Domain I (DI) is a central, eight-stranded β-barrel, which contains the only N-linked glycosylation site in WNV E. Domain II (DII) is a long, finger-like protrusion from DI and contains the highly conserved fusion peptide at its distal end. Domain III (DIII) adopts an immunoglobulin-like fold at the opposite end of DI, and is believed to contain a putative site for receptor attachment (9, 19, 50).

The hinge region between DI and DII is highly flexible, as the angle between these domains on DENV E rotates 27º during the transition from the immature to mature state (108). The hinge then rotates back 30º during the pre- to post-fusion domain rearrangement (13, 62, 67). Two x-ray crystal structures of similarly prepared WNV E protein also have distinct DI-DII hinge angles, differing by 5º (40, 72). Similarly, the position of DIII in relation to DI changes markedly during the fusion process. This is

necessary for the E protein to transition from a dimer, in which the fusion peptide is buried in the area between DI and DIII of its dimeric partner (61, 63, 84, 108), to a trimer, in which the fusion peptides of the three E proteins are grouped in an exposed conformation at the tip of the trimeric spike for insertion into the host endosomal membrane (13, 62, 67).

Virion structure. The mature flavivirus virion has a ~500Å diameter and consists of a single RNA genome surrounded by capsid protein, a lipid bilayer, and a shell of prM/M and E proteins (41-43, 65). In immature virions, three pairs of E and prM interact as trimers and form sixty heterotrimeric spiked projections with icosahedral symmetry (106, 107). In this formation, prM covers the hydrophobic fusion peptide and prevents adventitious insertion into host membranes during exocytosis (52, 104). As the immature virion traffics through the mildly acidic TGN, a dramatic rearrangement of the prM-E spikes occurs on the virion surface. This low pH induced transition causes the E proteins on immature virions to lie flat as antiparallel dimers on the surface of the virion (49), which allows for prM cleavage (52, 104). Mature flavivirus virions are covered by 90 anti-parallel E protein homodimers, which are arranged flat along the surface in a herringbone pattern with T=3 quasi-icosahedral symmetry (49, 65, 105).

The 180 E proteins on the surface of the mature virion exist in one of three different environments: the 2-, 3-, and 5-fold axes of symmetry (65). This is important in the context of antibody binding because, while one face of the E protein generally faces toward the virion membrane and the other outward, some epitopes are surface exposed only along certain axes of symmetry (42, 43, 57) or in certain states of maturation (18).

For example, the lateral ridge of DIII (DIII-LR) epitope is obscured at the 5-fold axis of symmetry, so a DIII-LR specific antibody can only bind to 120 of the 180 copies of E per virion (42, 71).

Similar to the maturation process, large rearrangements of the E proteins on the virion surface are required for fusion of the virion membrane to the host endosomal membrane. Lowered pH in the late endosome causes E protein dimers to dissociate and lift off of the surface of the virion (41), allowing fusion peptide insertion into the host membrane and E trimerization (13, 62, 67). It is hypothesized that the C-terminal stem-loop structures of the E protein then "zipper up" along the length of DII, pulling the spike back toward the surface of the virion and drawing the membrane of the endosome in proximity with the virion membrane. Apposition of the fusion loop and transmembrane domain of the E protein presumably causes the two membranes to fuse, completing the class II fusion process (62).

Anti-E antibodies. Studies in mice and other animals have established that humoral immunity is an essential component of the protective host response against flaviviruses, including WNV (reviewed in (78, 85)). B cells and secreted antibody were necessary for survival of mice after WNV inoculation (26, 27), and passive transfer of WNV-immune serum protected naive recipients from WNV challenge (28, 97). Moreover, pre-exposure prophylaxis and post-exposure therapy with WNV-specific monoclonal (MAb) or polyclonal antibodies conferred protection in both mice and hamsters (6, 7, 28, 64, 73, 95, 97, 99). Antibody neutralization can occur by blocking attachment to host cells, entry

of virions into cells, or the low pH-dependent fusion of the viral and host cell membranes (78).

The E protein of WNV is the principal target of neutralizing antibodies, although antibodies are also elicited against prM, NS1, NS3, and NS5 proteins (20, 100). Studies by several groups have shown that neutralization of WNV can occur after antibodies bind to a series of discrete epitopes on all three domains of the E protein (5, 23, 35, 73, 75, 86). The generation of large panels of mouse and human MAbs against epitopes spanning the WNV E protein has enhanced our understanding of the antibody response to WNV. The most potently neutralizing anti-WNV MAbs localize to the DIII-LR epitope. One well-characterized strongly neutralizing DIII-LR mouse MAb, E16, blocks infection primarily at a post-attachment step (71) and requires engagement of only a fraction of its epitopes on the surface of the virion (79). E16 acts by sterically hindering DIII from rotating over DI during the pH dependent fusion event in the late endosome (42, 98). While studies in WNV emphasize the potent neutralization potential of MAbs that bind to DIII-LR, studies with DENV have demonstrated the strong neutralization potential of MAbs that bind to additional DIII epitopes (14, 90, 93, 94).

Studies with anti-flavivirus MAbs have highlighted two other important functional aspects of antibodies: cross-reactivity and maturation sensitivity. MAbs directed against DIII-LR epitopes are specific to the virus they were generated against, and often even to the genotype within the specific species of flavivirus used for immunization (5, 14, 73, 86, 90, 93, 94). DIII-LR MAbs also equivalently neutralize WNV preparations that are fully mature, fully immature, and those with a mixture of maturity (68). Conversely, MAbs raised against the fusion loop of DII (DII-FL) are

generally cross-reactive amongst flaviviruses (21, 23, 34, 75), presumably due to the highly conserved amino acid sequence of this epitope, which is responsible for insertion into the host endosomal membrane during fusion (13, 62, 67). DII-FL MAbs can neutralize partially mature WNV but are unable to neutralize completely mature virus, which likely accounts for the fraction of virions resistant to neutralization by DII-FL MAbs when standard preparations of virus with mixed maturity are tested (68).

Studies of the human antibody response to WNV infection reveal that, in contrast to mice, antibodies that bind the DIII-LR epitope comprise a minor component of the humoral response in most individuals (74). Rather, the human anti-E repertoire appears to be skewed toward the DII-FL epitope in WNV and many flaviviruses (22, 74, 91, 99), which is somewhat surprising given that MAbs recognizing this epitope neutralize WNV poorly (75) and do not neutralize fully mature virus (68). However, the ability of DII-FL antibodies to cross-react against other flaviviruses (21, 23, 34, 75) could benefit people living in areas where multiple flaviviruses co-circulate by preventing heterologous infection.

Fc effector functions. Antibody neutralization of viruses is traditionally thought of as the ability to prevent infection by physically impeding either attachment or entry into cells (78). However, during infection in vivo the effector functions of the crystallizable fragment (Fc) of antibodies, specifically the ability to bind to complement (17) and Fc-γ receptors (70, 81), also can contribute to the protective effects of antibodies. Multiple experimental approaches have demonstrated the importance of Fc effector functions to antibody protection in vivo (8, 39, 60, 103). Several studies have compared protection by

MAb or polyclonal antibodies and lack of protection by the corresponding $F(ab')_2$ fragment(s) to conclude that Fc effector functions mediate protection (3, 11, 12, 35, 51, 58, 66). The conclusions of these comparisons have limitations because $F(ab')_2$ fragments are not recirculated by the neonatal Fc receptor (FcRn) and therefore have a much shorter half-life than antibodies in vivo. One study did control for $F(ab')_2$ recirculation effects by pre-adsorbing YFV with equimolar amounts of neutralizing MAb 2E10 or $F(ab')_2$, which was equally potent in vitro, then infecting mice intracerebrally. Higher mortality in $F(ab')_2$ treated mice at many concentrations tested suggested that the Fc portion of 2E10 was important for its protective effects in vivo (87).

Specific isotypes and subclasses of antibody are more effective at engaging Fc effector molecules. In this thesis, most studies are performed with IgG MAbs or purified polyclonal IgG, so this isotype will be the focus of the following material. In general, IgG must be bound to its cognate antigen to enable activation of Fc effector functions (31, 46, 69). Also, IgG molecules contain a single N-linked glycosylation on each heavy chain that is necessary for binding to both C1q and Fc-γ receptors (FcγR) (96).

C1q is the complement protein responsible for activation of the classical cascade of complement. When C1q binds to two or more IgG molecules that are bound to their antigen, a cascade of serine proteases are activated. C1r cleaves C1s, which then cleaves C2 and C4 into two fragments each. Combined, the C4b2a molecule forms the classical C3 convertase. This cleaves C3 molecules, causing a release of the C3a anaphylotoxin and covalent attachment of the C3b opsonin onto the virion surface, activating the immune system and marking the virion for phagocytosis, respectively (reviewed in (82)). Opsonization of antigens with C3b also increases activation of the humoral immune

response via complement receptors CD21 (CR2) and CD35 (CR1) by enhancing antigen presentation on macrophages and dendritic cells and dramatically lowering the threshold of activation for B cells (16). Other complement molecules, such as mannose binding lectin (MBL), can engage antigen-bound IgG and initiate the complement cascade via C1q independent bypass pathways (2). Generally, human IgG3 > IgG1 >> IgG2 (10) and mouse IgG2b > IgG2a = IgG2c > IgG3 > IgG1 (33) at binding to C1q, although some murine IgG1 molecules can fix complement while others cannot (29, 30).

In vitro studies have demonstrated the ability of C1q to enhance the neutralizing effects of MAbs. Comparing the addition of serum from wild type and *C1q*$^{-/-}$ mice or by adding purified C1q to neutralization assays demonstrated the ability of C1q to reduce the 50% effective concentration (EC_{50}) of various anti-WNV MAbs. The use of MAb E16 with an A330L mutation in the heavy chain, which abolished C1q binding, confirmed the shift in EC_{50} was C1q dependent. This effect was due to cross linking of antibodies on the virion surface, which decreased the amount of MAb required for neutralization (59). In related studies, in vitro neutralization by the anti-vaccinia virus MAb B126 was only detectable upon the addition of purified C1q. The importance of complement for the function of B126 was highlighted in vivo, as it prevented weight loss in vaccinia challenged mice, but this effect was diminished in mice depleted of complement activity by treatment with cobra venom factor (8).

The other major Fc effector mechanism of IgG is the engagement of FcγRs, which can be activating (FcγRI, CD64; FcγRIII, CD16; FcγRIV) and inhibitory (FcγRIIb, CD32). Generally, FcγRI > FcγRIV > FcγRIIb ≈ FcγRIII in their affinity for IgG molecules, although different FcγRs bind preferentially to different antibody isotypes

(70). Activating FcγRs are expressed on the surface of cells, and most associate with an intracellular signaling molecule, the common gamma chain of activating FcγRs (FcRγ). All activating FcγR intracellular signaling occurs via immunoreceptor tyrosine-based activation motifs (ITAMs). Various immune cell types express different combinations of activating FcγRs, and concordantly ligation of these FcγRs can trigger many effects: phagocytosis of immune complexes, antibody dependent cellular cytotoxicity (ADCC), and downstream signaling that activates cells or stimulates the production and release of cytokines. The inhibitory FcγRIIb is a transmembrane molecule with an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM), which is mainly responsible for tempering the immune response in states of high activation, especially by increasing the activation threshold in B cells to prevent the overproduction of antibodies (reviewed in (81)).

Previous studies have implicated FcγRs as crucial to in vivo protection by antiviral MAbs. Three non-neutralizing MAbs individually reduced murid herpesvirus 4 titers in the lungs of wild type but not *FcγR*$^{-/-}$ mice, which lacked both activating and inhibitory FcγR (103). An L234A, L235A variant of the neutralizing anti-human immunodeficiency virus (HIV) b12 MAb, which cannot bind to FcγRs or C1q, could not suppress chimeric simian immunodeficiency virus-HIV plasma titers in macaques as effectively as wild type b12. However, a K322A variant of b12, which lacks binding to C1q but retains FcγR binding, reduced titers similarly to wild type b12, indicating a requirement of FcγR engagement for this protective activity (39). Pre-treatment of mice with neutralizing MAb 667 against a different retrovirus, a variant of the Friend murine leukemia virus, enhanced many aspects of the adaptive immune response to infection.

Dendritic cell uptake of antibody-virion immune complexes was inhibited by an FcγR blocking MAb as well as the use of $F(ab')_2$ fragments rather than MAb to create immune complexes prior to infection (eliminating concerns of lack of recirculation by FcRn) (60).

Rationale. The studies described in this thesis examined how the antibody response to WNV is protective despite being skewed away from the most potently neutralizing epitope (DIII-LR) toward a poorly neutralizing epitope (DII-FL). The first section addresses the hypothesis that WNV infection may indeed induce strongly neutralizing antibodies that simply go undetected by current epitope-specific diagnostics, which utilize recombinant E proteins. For these studies, two WNV-specific human MAbs that were isolated from B cell populations of convalescent patients were characterized in detail. These MAbs were initially identified as being able to bind WNV virions and subviral particles but not recombinant E protein, yet they strongly neutralized WNV infection in vitro and protected mice against lethal infection in vivo. This indicated their potential for revealing previously unappreciated epitopes on the virion.

Given the rapid spread of WNV throughout the United States and into Canada, it was surprising that so few human cases have been reported in Latin America. One hypothesis for this discrepancy was that the high prevalence of DENV infections and frequency of YFV vaccination in this region may result in "susceptible" individuals having poorly-neutralizing cross-reactive antibodies against WNV that are not detected by standard neutralization assays. However, poorly neutralizing mouse MAbs had been shown to protect mice from WNV infection in vivo, a phenomenon that also had been demonstrated in several other in vivo models of virus infection. Therefore, the second

part of this thesis was aimed at determining whether poorly neutralizing MAbs and polyclonal antibodies protect against WNV in vivo, and if so whether this was due to their Fc effector functions.

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Chapter II

Human Monoclonal Antibodies Induced by Natural Infection Against West Nile Virus Neutralize at a Post-Attachment Step

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INTRODUCTION

West Nile encephalitis virus (WNV) is a positive polarity single-stranded RNA virus of the Flavivirus genus within the *Flaviviridae* family. Other members of this genus that cause significant human disease include dengue (DENV), St. Louis encephalitis (SLEV), Japanese encephalitis (JEV), yellow fever (YFV), and tick-borne encephalitis (TBEV) viruses. Flaviviruses are translated as a single polypeptide, which is then cleaved by host and viral proteases into three structural (capsid [C], pre-membrane [prM], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (reviewed in (42, 43)).

WNV cycles in nature between several species of birds and *Culex* mosquitoes, with humans and other mammals as dead-end hosts (25, 62). Infection causes syndromes ranging from a mild febrile illness to severe encephalitis and death (13, 72). WNV has spread globally and causes outbreaks with thousands of severe human cases annually in the United States. Age greater than 55, a compromised immune status, and a CC5Δ32 genotype have been associated with more severe disease (15, 20). There is currently no approved vaccine or therapy for WNV infection.

The mature WNV virion has a ~500Å diameter and consists of a single RNA genome surrounded by capsid protein, a lipid bilayer, and a shell of prM/M and E proteins (31, 55). X-ray crystallography studies have elucidated the three-domain structure of the flavivirus E protein (30, 48, 50, 58, 67). Domain I (DI) is a central, eight-stranded β-barrel, which contains the only N-linked glycosylation site in WNV E. Domain II (DII) is a long, finger-like protrusion from DI and contains the highly conserved fusion peptide at its distal end. Domain III (DIII) adopts an immunoglobulin-

like fold at the opposite end of DI, and is believed to contain a putative site for receptor attachment (6, 8, 40).

Within an infected cell, progeny WNV are assembled initially as immature particles. In immature virions, three pairs of E and prM interact as trimers and form sixty spiked projections with icosahedral symmetry (85, 86). Exposure to mildly acidic conditions in the trans-Golgi secretory pathway promotes virus maturation through a structural rearrangement of the E proteins and cleavage of prM to M by a furin-like protease (41, 83). Mature WNV virions are covered by 90 anti-parallel E protein homodimers, which are arranged flat along the surface in a herringbone pattern with quasi-icosahedral symmetry (55).

Upon binding to poorly characterized cell surface receptors, internalization of WNV is believed to occur through receptor-mediated, clathrin-dependent endocytosis (1, 79, 80). After trafficking to Rab5- and/or Rab7-positive endosomes (38, 79), the mildly acidic pH within the lumen of the endosome induces structural alterations in the flavivirus E protein (7, 49), which includes changes in its oligomeric state (7, 49, 77). During this process, also known as type II fusion, the hydrophobic peptide on the fusion loop of domain II of the E protein inserts into the endosomal membrane, thus physically joining the host and viral membranes, which allows the infectious RNA genome to enter the cytoplasm (32, 33).

Humoral immunity is an essential component of the protective host response against flaviviruses, including WNV (reviewed in (64, 68)). Studies by several groups have shown that neutralization of WNV can occur after antibodies bind to a series of discrete epitopes on all three domains of the E protein (3, 12, 22, 59, 61, 71). To date, the

most potently neutralizing MAbs localize to an epitope on the lateral ridge of DIII (DIII-lr). One well-characterized strongly neutralizing mouse MAb, E16, blocks infection primarily at a post-attachment step (57) and requires engagement of only a fraction of its epitopes on the surface of the virion (66). Studies of the human antibody response to WNV infection reveal that, in contrast to mice, antibodies that bind the DIII-lr epitope comprise a minor component of the neutralizing humoral response in most individuals (60).

In this study, we characterized two strongly neutralizing novel human MAbs (CR4348 and CR4354) that were selected from an antibody phage display library constructed from B cells of subjects that survived WNV infection (78). We demonstrate that both MAbs are WNV-specific, bind weakly to recombinant or yeast surface-displayed E proteins, exhibit pH-sensitive binding to viral particles, and protect against lethal infection in mice. Our experiments suggest these human MAbs map to distinct epitopes and neutralize infection at a post-attachment stage, likely by inhibiting the acid-catalyzed viral fusion step.

MATERIALS AND METHODS

Preparation of virus, subviral particles, and pyrene-labeled virus. WNV strain 3000.0259, which was isolated in New York in 2000 (16), was used to generate stocks of passage 2 (4.2 x 10^7 PFU/ml) and passage 3 (2.0 x 10^7 PFU/ml) virus after propagation in C6/36 *Aedes albopictus* cells. The propagation of wild type and mutant WNV from the New York 1999 (NY99ic) infectious clone (5) are detailed below. WNV subviral particles (SVP) were generated after transfection of BHK21-15 cells with a pcDNA3.1 plasmid expressing premembrane (prM) and envelope (E) genes (36) from the NY99 WNV strain using FuGENE HD (Roche) according to the manufacturer's instructions. Supernatants containing SVP were collected 48 hours after transfection, filtered through a 0.2 μm filter, and stored aliquotted at -80ºC.

Labeling of WNV with the fluorescent probe pyrene was performed essentially as described for alphaviruses (73, 81). Briefly, BHK21-15 cells cultured in the presence of 15 μg/ml of 16-(1-pyrenyl)-hexadecanoic acid (Invitrogen) were infected with WNV at an MOI of 4. At 24 hours post-infection, the medium was harvested, clarified by low-speed centrifugation and pyrene-labeled WNV particles were pelleted by ultracentrifugation in a Beckman type 19 rotor for 15 hours at 48,500 x g at 4°C. The virus particles were further purified on an Optiprep (Axis-Shield) density (15-55% w/v) gradient by ultracentrifugation in a Beckman SW41 rotor for 18 hours at 100,000 x g at 4°C. The infectious titer was determined by $TCID_{50}$ analysis and the protein concentration was measured by micro-Lowry analysis.

Monoclonal antibodies (MAbs). Unless otherwise specified, all antibodies used were protein A-purified and of the human IgG1 subclass. Humanized E16 (Hu-E16) was

generated from a mouse MAb after genetic engineering as described previously (59). CR4348, CR4354, and CR4293 (anti-prM) were selected from scFv-phage display libraries constructed from peripheral blood lymphocytes isolated from three human patients who survived neuroinvasive WNV disease (78). Construction of the libraries, selections with the libraries and reformatting of scFv-phage into full-length IgG1 molecules has been previously described in detail (78). WNV E24 (mouse IgG2a) localizes to the DIII-lr epitope (59) and was purified by protein A affinity chromatography. Control non-WNV reactive anti-FITC and anti-DENV1 E50 human IgG1 were gifts of S. Johnson (Macrogenics, Rockville, MD).

Neutralization assays. (a) Plaque Reduction Neutralization Test (PRNT). In many experiments, the neutralizing activity of MAbs was determined using a $PRNT_{50}$ analysis. Briefly, serially diluted MAbs were mixed 1:1 with 10^2 PFU of WNV in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Omega Scientific) and incubated one hour at 37ºC. The WNV-MAb mixture was then added to individual wells of a 6-well tissue culture plate in duplicate or triplicate with either Vero or BHK21-15 cells. Viral adsorption proceeded for one hour at 37ºC, followed by an overlay with 1% low-melt agarose (SeaPlaque) in α-MEM and 4% FBS. After solidification, plaques were visualized three to four days later following fixation with 2 ml of 10% formaldehyde, removal of agarose plugs, and staining with 1% (w/v) crystal violet in 20% (v/v) ethanol. Plaques were counted and then normalized to the average of six control wells in which WNV was mixed with DMEM containing 10% FBS and no antibody.

(b) Pre- and post-attachment neutralization assays. To assay for post-attachment neutralization, a PRNT assay was completed essentially as above with the following

modifications. All solutions and Vero cells were pre-chilled to 4ºC, then 10^2 PFU of WNV was added to each well of cells and viral adsorption allowed for one hour at 4ºC. Wells were then washed thrice with media, and MAb at the specified concentrations was added. Virion-antibody complexes were allowed to form for one hour at 4ºC followed by three washes with chilled media. Cells were then warmed to 37ºC and the PRNT assay was completed as described above. In parallel, a PRNT assay with all cells and solutions at 4ºC was performed in which MAb and WNV were mixed for one hour at 4ºC prior to addition to cells (pre-attachment assay).

(c) Reporter Virus Particle (RVP) Assay. WNV and mutant RVP were generated as described previously (65, 66). Separate plasmids expressing the wild type prM-E genes and the capsid (C) gene of WNV were transfected into a BHK cell that stably propagates a WNV replicon expressing GFP. In some experiments, the prM-E plasmid was mutated using the QuikChange site directed mutagenesis kit (Stratagene) to introduce specific amino acid substitutions. In other experiments, RVP were produced from 293T cells at various stages of maturation (immature, partially mature, or fully mature) according to published protocols (56). Supernatants containing RVP were harvested 48 hours after transfection, filtered through a 0.2 μm filter, and stored aliquotted at -80ºC. RVP were incubated with serial dilutions of MAb under conditions of antibody excess at room temperature (RT) for one hour. Subsequently, MAb-RVP mixtures were added to Raji-DCSIGNR cells, which stably express the DC-SIGNR attachment factor (14), and were incubated at 37ºC for 48 hours. Infected cells were assayed for GFP expression using a BD FACSArray flow cytometer. Alternatively human CD32A (Fc-γRIIA) expressing K562 cells were used to assay for antibody dependent enhancement of infection.

Yeast surface display of WNV E proteins. The generation of yeast that express the WNV E protein ectodomain (amino acid residues 1-415) or DIII (residues 296-415) has been described previously (59). Yeast expressing WNV E or DIII were washed in PBS, 2% BSA, and 0.025% NaN_3, incubated with primary MAbs (50 μg/ml) for 30 minutes on ice, washed thrice, mixed with a 1:500 dilution of AlexaFluor 647 conjugated goat anti-human antibody (Molecular Probes), washed again, and processed on a BD FACSArray flow cytometer.

Antigen capture and solid-phase ELISA. Nunc MaxiSorp polystyrene 96-well plates were either coated overnight at 4ºC with murine DIII-lr MAb (10 μg/ml) or for one hour at 37ºC with soluble recombinant WNV E protein ectodomain (10 μg/ml, generated as described in (57)) in a pH 9.3 carbonate buffer. Plates were washed thrice in ELISA wash buffer (PBS with 0.02% Tween 20) and blocked for one hour at 37ºC with ELISA block buffer (PBS, 2% bovine serum albumin, and 0.02% Tween 20). On plates coated with murine DIII-lr MAb, SVP or WNV infectious virions (wild type or mutant) were captured for one hour at RT. Subsequently, plates were rinsed five times in wash buffer and then incubated with anti-WNV or control human IgG1 (10 μg/ml in block buffer) in triplicate for one hour at RT. Plates were washed five times and then incubated with biotinylated rabbit anti-human IgG antibody (1:1250 dilution, Southern Biotech) for one hour at RT in blocking buffer. Plates were washed again five times and then sequentially incubated with 2 μg/ml of horseradish peroxidase conjugated streptavidin (Vector Laboratories) and tetramethylbenzidine (TMB) substrate (Dako). The reaction was stopped with 2N H_2SO_4 and emission (450 nm) was read on an iMark microplate reader (BIORAD). In the pH-dependent ELISA, plates were washed four times with wash buffer

and once with indicated pH buffer (150 mM NaCl, 0.05% Tween 20, 50 mM MES [pH 6.0] or wash buffer [pH 7.4]) after trapping of SVP. The plate was incubated for 30 minutes at RT with indicated pH buffer, and then washed five times in wash buffer followed by normal completion of capture ELISA protocol.

Western blots. Recombinant WNV E protein, SVP, or infectious WNV was diluted in 2X SDS loading buffer with or without β-mercaptoethanol (5% v/v) and incubated at RT or 95ºC for 10 minutes as indicated. Samples were loaded into wells of a NuPAGE (Invitrogen) 4-12% Bis-Tris gradient gel and electrophoresed. Protein was transferred to a polyvinylidene fluoride (PVDF) transfer membrane using the iBlot system (Invitrogen). Membranes were rinsed in PBS, 0.05% Tween 20 (wash buffer) while gently shaking for 10 minutes at RT, then blocked overnight with block buffer (5% dry milk in wash buffer) shaking at RT. After five 10 minute washes, membranes were stained with primary human MAb (1 μg/ml diluted in block buffer with normal goat serum added at 1:250 dilution) for one hour at RT. After five additional washes, membranes were incubated with horseradish peroxidase conjugated goat anti-human antibody (Sigma, diluted 1:5000 in block buffer) for one hour. Membranes were then washed five times for 10 minutes in wash buffer and developed using ECL reagent (Amersham).

Generation of neutralization escape mutants. WNV was incubated with 25 μg/ml of CR4348 or CR4354 for one hour at RT in DMEM. The mixture was added to Vero cells in a 6 well plate at a multiplicity of infection (MOI) of 1. After infection for 2 hours at 37ºC, wells were washed thrice with DMEM and fresh media containing 25 μg/ml of MAb was added. Virus growth under antibody selection was allowed for 48

hours at 37 ºC. At each passage, half of the supernatant was mixed 1:1 with a 50 μg/ml of MAb for one hour. The remaining half of the supernatant was aliquotted and stored at -80ºC. After three passages under MAb selection, virus-containing supernatants were tested by PRNT for escape from neutralization by CR4348 or CR4354. After confirmation of the escape phenotype, an aliquot of the supernatant was used in a Vero cell plaque assay under MAb selection. Plaques were visualized by overlaying with neutral red and sterile glass Pasteur pipettes were used to isolate resistant virus from single plaques. Plaque-purified virus was amplified under MAb selection (25 μg/ml) overnight at 37ºC. Vero cells were scraped from wells and total cellular RNA isolated using an RNEasy kit (Qiagen). cDNA was amplified using a reverse primer (2501R: 5'-TGCCGGCTGATGTCTATGG -3') in the WNV NS1 gene, and served as a template for polymerase chain reaction (PCR) amplification of the prM and E genes using forward (454F: 5'-AGCGTAGGAGCAGTTACCC-3') and the 2501R reverse primers. The prM and E genes were then directly sequenced from gel purified PCR products and the neutralization escape mutant sequence was compared to the sequence of the laboratory stock WNV that was passaged and plaque-purified in parallel in the absence of MAb selection.

WNV infectious cDNA clone and mutant generation. The two plasmid WNV NY99 cDNA clone (36) was used to create wild type and mutant infectious WNV. Single amino acid substitutions were introduced into the pWNAB plasmid by site directed mutagenesis. Wild type and mutant pWNAB (encoding nucleotides 1-2495 of the WNV genome) and wild type pWNCG (nucleotides 2495-11,029) plasmids were grown in SURE-2 supercompetent *E. coli* (Stratagene) at RT. Each plasmid was digested with

XbaI and NgoMIV restriction endonucleases and the resultant ~5.2 and 8 kilobase fragments of pWNAB and pWNCG, respectively, were gel purified and ligated with T4 DNA ligase (Invitrogen) at 4ºC overnight. The reactions were then heat-inactivated, digested with XbaI to linearize the DNA, treated with proteinase K, extracted twice with a phenol and chloroform, and precipitated with ethanol at -20ºC overnight. All DNA was used as a template for in vitro DNA-dependent RNA transcription with the AmpliScribe T7 kit (Epicentre) with the addition of $m^7G(5')ppp(5')A$ cap analog (New England BioLabs). Transcription reactions were run at 37ºC for 5 hours and the reaction was then electroporated (3 pulses at 850V, 25μF, ∞Ω) into BHK21-15 cells. Cells were added to a T75 tissue culture flask in DMEM with 10% FBS and observed for the onset of cytopathic effects (CPE). Once CPE were observed, virus-containing supernatant was collected, cellular debris pelleted, and supernatant aliquots frozen at -80ºC. The cells remaining in the T75 were harvested and used as a source of viral RNA to confirm that the desired mutant sequence was retained.

Structural Analysis. The coordinates for the WNV E protein (RCSB accession number 2HG0) were divided by domains and fit onto the cryoelectron microscopy structure for the mature Dengue virus (RCSB accession number 1K4R) using CCP4MG to create a model of the WNV E protein dimer. Distances were calculated and figures prepared using PyMol (http://www.pymol.org).

Mouse experiments. Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Three to four week-old outbred NIH Swiss mice (Harlan) were infected by intraperitoneal (IP) injection with WNV NY99ic diluted in Hank's Balanced Salt

Solution containing 1% heat-inactivated FBS. For antibody protection studies, one day prior to infection mice were treated by IP injection with 50 μg of indicated MAb or vehicle control diluted in 100 μl PBS. Mice were monitored daily for 21 days for mortality and were euthanized when moribund. In some experiments, passive transfer of MAbs was performed with five week-old C57BL/6 mice (Jackson Laboratories) as described previously (59).

Fusion assay. Fusion of pyrene-labeled WNV with liposomes was monitored continuously in a Fluorolog 3-22 fluorometer (BFi Optilas), at excitation and emission wavelengths of 345 nm and 475 nm, respectively. Liposomes (large unilamellar vesicles with a diameter of ~200 nm) were prepared by a freeze/thaw-extrusion procedure as described previously (73). Liposomes consisted of a mixture of phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) prepared by transphosphatidylation of egg PC, and cholesterol in a molar ratio of 1:1:2. The lipids were obtained from Avanti Polar Lipids. The concentration of phospholipids was determined by phosphate analysis.

Pyrene-labeled WNV (1.24 μg of total viral protein) and liposomes (final concentration, 200 μM phospholipid) were mixed in a final volume of 665 μl in 5 mM Hepes, 150 mM NaCl, 0.1 mM EDTA, pH 7.4 (HNE) under continuous stirring in a temperature-controlled cuvette at 37°C. At t = 0 seconds, the medium was acidified by addition of 35 μl 0.1 MES, 0.2 M acetic acid, pre-titrated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence value. The 100% value was obtained through the addition of 35 μl 0.4 M octaethyleneglycol monododecyl ether (Fluka Chemie AG) to achieve an infinite dilution of the probe. The extent of fusion was determined 60 seconds after

acidification. To determine the influence of the MAbs on membrane fusion, pyrene-labeled WNV was incubated with different concentrations of MAbs for one hour at RT prior to mixing with liposomes.

Statistical Analysis. All data were analyzed using Prism software (GraphPad Prism, San Diego, CA). Kaplan-Meier survival curves were analyzed by the log-rank test. For neutralization assays an unpaired student's T-test was used to determine significance. For ELISA data a paired student's T-test was used to determine significance.

RESULTS

Characterization of strongly neutralizing human MAbs. Previous studies that mapped the epitope specificity of inhibitory antibodies in convalescent human serum suggested that only a fraction of WNV-infected patients developed antibodies against the strongly neutralizing epitope on DIII-lr of the E protein (60). Consistent with this, few DIII-specific human MAbs were isolated from phage display single chain variable fragment (scFv) libraries from infected patients (78) and none were selected from very large libraries of pooled uninfected donors (22). As human convalescent serum retains strong neutralizing activity, we hypothesized that antibodies with distinct specificities must contribute to the inhibitory activity.

Antibody-phage display libraries constructed from peripheral blood lymphocytes of three convalescent patients after WNV infection were screened for reactivity on WNV antigen as described previously (78). All selected monoclonal phage specifically bound a preparation of inactivated WNV and immobilized prM-E containing subviral particles (SVP). SVP, in addition to containing prM/M proteins, display 60 E protein homodimers in a lipid bilayer (18) whereas virions have 90 E protein homodimers in a distinct icosahedral arrangement (39, 55). Two MAbs, CR4348 and CR4354, which demonstrated strong neutralizing activity in pilot functional assays, tested negative for binding to recombinant E protein by ELISA (data not shown); in contrast, other neutralizing human MAbs that were identified in the screen and previously characterized (e.g., CR4374 (60, 78)) readily bound recombinant E protein.

In both plaque reduction (PRNT) and WNV reporter virus particle (RVP) assays CR4348 ($PRNT_{50}$ = 536 ng/ml; RVP EC_{50} = 146 ng/ml) and CR4354 ($PRNT_{50}$ = 88

ng/ml; RVP EC_{50} = 26 ng/ml) inhibited infection strongly, albeit slightly less than that achieved with a humanized version of a therapeutic WNV type-specific mouse MAb, E16 (Hu-E16) that maps to the DIII-lr epitope (59) (**Fig 1A and 1B**). CR4348 and CR4354 appeared distinct from Hu-E16 as they did not efficiently recognize the ectodomain of E protein when expressed as purified protein or displayed on the surface of yeast (**Fig 2A and 2B**). These neutralizing human MAbs also efficiently neutralized variant WNV RVP composed of E proteins encoding a T332K mutation that abrogates neutralization of virtually all DIII-lr MAbs (**Fig 1C** and (3, 59)). Additionally, both human MAbs likely do not recognize linear epitopes as they did not identify prM, M, or E by Western blot under reducing or non-reducing conditions (**Fig 2C**).

Because CR4348 and CR4354 neutralized WNV but did not appear to recognize recombinant E protein, we speculated they might inhibit infection by binding to an epitope on prM that was not be detected by Western blot. To test this, we took advantage of a series of WNV RVP preparations that differ with respect to the efficiency of the maturation process; similar preparations were used previously to define differential neutralizing activity of anti-WNV MAbs generated in mice (56). WNV RVPs were produced as fully mature (in cells over-expressing the furin protease), partially and heterogeneously mature (standard conditions) or largely immature (in the presence of NH_4Cl, which inhibits furin cleavage). These three types of RVP differ in their content of prM ranging from virtually none (mature) to >95% (immature). CR4348 and CR4354 neutralized prM-containing or absent particles equivalently (**Fig 3**), eliminating the pr peptide as part of the epitope for either MAb.

An alternative possibility was that CR4348 and CR4354 do not bind a viral protein but instead inhibit WNV infection by binding a host cell surface receptor and blocking attachment and/or entry. However, if the neutralizing MAbs CR4348 and CR4354 recognized a structural protein on the virion, they should enhance infection in Fc-γ receptor expressing cells when the stoichiometry of binding falls below the neutralization threshold (51, 66). Addition of sub-neutralizing concentrations of CR4348 and CR4354 to K562 cells that display human CD32 (Fc-γRIIA) enhanced infection in a manner that was analogous to that observed with Hu-E16 (**Fig 1D**). This experiment suggests that CR4348 and CR4354 recognized a protein on the surface of the infectious virion.

To corroborate these findings, we used a capture ELISA to measure direct binding of CR4348 and CR4354 to SVP and infectious WNV virions. Notably, CR4348 and CR4354 bound both to SVP and infectious virus (**Fig 2D**, $P \leq 0.002$). However, CR4348 and CR4354 did not bind to DENV particles, consistent with their inability to neutralize these viruses in a plaque reduction assay (data not shown). Thus, the biochemical and functional analyses suggested that CR4348 and CR4354 neutralize WNV by virtue of their ability to bind an epitope on one of the viral structural proteins (M or E) that requires a specific oligomeric arrangement present on the virions or SVP.

Neutralization by CR4348 and CR4354 occurs primarily at a post-attachment step. Antibody neutralization of enveloped viruses may occur by inhibiting receptor attachment, internalization, and/or endosomal fusion (63, 88). To determine the stage of the viral entry pathway at which CR4348 and CR4354 inhibit infection, we performed pre- and post-attachment neutralization assays (11, 29, 57). CR4348 and CR4354 were

incubated with WNV before or after virus binding to a monolayer of Vero cells and infection was measured by a plaque reduction assay. As expected, CR4348 and CR4354 efficiently neutralized infection when pre-mixed with virus (**Fig 4A**). Both MAbs also inhibited WNV infection when added after virus adsorption to the cell surface, indicating that at least part of their neutralizing activity was at a post-attachment step of the viral life cycle. When added after attachment, CR4354 was more potent than CR4348 in neutralizing infection. Importantly, no pre- or post-attachment neutralization was detected with a non-binding isotype control, and a largely post-attachment pattern of inhibition was seen with Hu-E16 (**Fig 4A**) as observed previously (57).

pH sensitivity of CR4348 and CR4354 binding. Exposure of flavivirus virions or SVP to acidic pH in solution or in the endosome prompts a rapid structural rearrangement (7, 19, 49, 77). Given that CR4348 and CR4354 preferentially recognize epitopes present on virions and SVP, but not on recombinant E proteins (**Fig 2**), we hypothesized that changes in the oligomeric arrangement of E that are associated with shifts in pH might alter immunoreactivity. To test this, we used our SVP capture ELISA, exposing particles to different pH (6.0 or 7.4) conditions for 30 minutes prior to pH normalization (pH 7.4) and incubation with MAbs. Notably, our control neutralizing MAb, Hu-E16, did not show pH-sensitive binding (**Fig 4B**). In contrast, exposure of SVP to pH 6.0, which should promote E protein rearrangement and irreversible trimer formation, significantly reduced binding of both CR4348 and CR4354 ($P \leq 0.003$).

CR4348 and CR4354 inhibit WNV fusion. Since both CR4348 and CR4354 have the capacity to neutralize WNV infection at a post-attachment step, we investigated whether these mAbs would also affect WNV fusion. To evaluate this, we utilized a model

liposome fusion assay with pyrene-labeled virus that has been previously used to monitor the fusion dynamics of TBEV (9, 19, 75-77). Briefly, pyrene-labeled WNV was pre-incubated with different concentrations of MAbs for one hour at room temperature and then mixed with liposomes at 37°C. Fusion was triggered by acidification of the mixture to pH 6.3, which is the optimal pH for WNV fusion (B. Moesker, J. Wilschut and J. Smit, unpublished observations). In the absence of antibodies or in the presence of 50 nM (7.5 μg/ml) of a non-binding control MAb, fusion was essentially complete within seconds upon acidification. In contrast, pre-incubation of the virus with CR4348 or CR4354 potently inhibited fusion activity (**Fig 4C**). Both MAbs reduced the extent of fusion in a dose-dependent manner (**Fig 4D**). CR4354 inhibited fusion at concentrations of 1 nM (or 0.15 μg/ml) and above, whereas for CR4348 a minimal concentration of 10 nM (or 1.5 μg/ml) was required. This difference in potency was also reflected in the maximum level of fusion inhibition at saturating antibody concentrations. CR4354 and CR4348 neutralized ~85% and ~65% of the virus particles, respectively (**Fig 4D**). Thus, in the model liposome assay, CR4348 and CR4354 can block low-pH-catalyzed fusion of the majority of WNV virions; this supports the idea that these MAbs act at a post-attachment step. Nonetheless, even under saturating antibody concentrations, a fraction of the virus particles resists complete inhibition by these MAbs, although the rate of fusion of the residual fractions are substantially lower than in the absence of antibody.

Generation of neutralization escape mutants. Because CR4348 and CR4354 recognized a determinant on the WNV virion or SVP, which is not readily apparent on recombinant or yeast displayed forms of E, we generated neutralization escape mutants to further define their epitopes. After three sequential virus passages on Vero cells under

CR4348 or CR4354 selection (25 μg/ml), WNV was no longer neutralized by these MAbs in plaque reduction assays (**Fig 5A**). To determine the mutations that conferred the escape phenotype, RNA sequences obtained from plaque-purified escape variants were compared to wild type WNV sequence derived from virus passaged in parallel in the absence of antibody selection. All (5 of 5) sequences from CR4348 escape variants contained the same single nucleotide change encoding a T208I mutation in the E protein; in addition, two of the five sequences also had an independent H246Y mutation in the E protein. In contrast, all (15 of 15) CR4354 escape variants contained a single nucleotide mutation, encoding a K136E mutation in the E protein; no other nucleotide changes were observed in any of the CR4354 variants.

To establish that these amino acid substitutions conferred the neutralization escape phenotypes observed, we utilized two reverse-genetic systems. First, RVP with single amino acid mutations were generated and analyzed for MAb neutralization. Whereas Hu-E16 neutralized all mutant and WT RVP equivalently, the T208I and K136E RVPs were not efficiently neutralized by CR4348 and CR4354, respectively, even at concentrations of 15 μg/ml of MAb (**Fig 5B**). H246Y RVP were neutralized by CR4348 but only at the highest doses of antibody tested; the concentration at which 50% inhibition (EC_{50}) occurred was increased by > 31-fold ($P < 0.02$) compared to the wild type RVP (see **Fig 1B**).

As an independent confirmation, we introduced these substitutions into a wild type infectious New York 1999 WNV cDNA clone (NY99ic) (5) to generate mutant viruses. Analogous results were obtained with all genetically engineered mutant WNV and MAbs using a plaque reduction assay on BHK21-15 cells (**Fig 5C**). Thus,

experiments with the RVP and infectious cDNA clone confirmed the sequencing results, and suggest that CR4348 and CR4354 likely bind distinct epitopes on WNV E protein. To directly determine the contributions of these amino acids to MAb binding, we used the mutated NY99ic WNV in a capture ELISA (**Fig 5D**). As expected, Hu-E16 recognized all three variant viruses. CR4354 failed to bind to WNV with a K136E mutation but did recognize the other viruses, establishing that a change in K136 confers a loss-of-binding phenotype. In contrast, no decrease in CR4348 binding was observed for the single T208I or H246Y variants or a double T208I/H246Y variant (data not shown). Thus, these two individual mutations, which strongly impact CR4348 neutralization, do not alter MAb binding in the capture ELISA.

To gain a better understanding of why CR4348 and CR4354 recognized intact virions or subviral particles but not recombinant E proteins, we mapped the residues that conferred escape from MAb neutralization onto the existing WNV E protein crystal structures (30, 58). K136, T208, and H246 are all solvent-accessible residues **(Fig 6A)**. K136 is located at the end of DI adjacent to the hinge between DI and DII. This hinge rotates ~20-30º during structural rearrangement of the E protein associated with low pH-induced viral fusion in late endosomes (7, 49). The flexibility of the DI-DII hinge may be less dynamic on an icosahedral virion or SVP compared to soluble recombinant protein, possibly explaining the differential reactivity of the CR4354 epitope. In contrast, the conformationally sensitive nature of CR4348 binding may be due to preferential reactivity with WNV E protein dimers. T208 and H246 are present in DII and, although relatively distant (~43Å) within an individual E protein monomer, are separated by ~19Å across the dimer interface, which is within the spatial limits of an antibody footprint **(Fig**

6B). It is important to note that in contrast to TBEV and DENV E proteins, which readily form dimers in solution, soluble WNV E protein is largely monomeric (30, 58). Consistent with the mapping and binding data that suggests the two human MAbs recognize distinct epitopes, CR4348 efficiently neutralized the K136E WNV variant and CR4354 inhibited infection of the T208I and H246P variants (data not shown).

Given this mapping data, we hypothesized that CR4348 might recognize oligomeric forms of E protein that are not prevalent in our purified recombinant preparation. As mentioned, ELISA plates coated with WNV E protein overnight at 4ºC showed no immunoreactivity with either CR4348 or CR4354 (**Fig 2B**). However, coating plates with E protein at 37ºC resulted in a modest, yet significant signal by CR4348, possibly due to oligomeric interactions between E proteins that occur more favorably at a higher temperature, prior to adsorption (**Fig 2B**).

Phenotype of WNV variants in cell culture. Comparison of E protein amino acid sequence of WNV isolates that varied geographically and temporally showed that T208 and H246 were completely conserved. K136 was completely conserved among lineage 1 WNV isolates, but varied in the less virulent lineage 2 and 3 strains with alanine and serine substitutions, respectively (data not shown). To evaluate whether the mutations associated with neutralization escape of CR4348 and CR4354 were functionally important for virus replication and affected viral virulence, growth analyses in BHK21-15 cells was performed. The plaque morphology of the three mutant viruses (K136E, T208I, and H246Y) in BHK21-15 cells was compared to that of the wild type WNV New York 1999 strain. The wild type, K136E and T208I variants had similar large plaque morphology whereas the H246Y mutant had a small plaque phenotype (data not shown).

In vivo protection studies. Passive transfer of neutralizing MAbs against WNV confers protection against disease in mice (22, 59, 61, 69, 78) and hamsters (53, 54). To evaluate the potency of the human MAbs, protection studies were performed in wild type C57BL/6 mice after infection with WNV-NY99ic. Analogous to results with Hu-E16 (59), prophylaxis with low (1.4 and 0.4 μg, respectively) doses of CR4348 and CR4354, completely protected mice against lethal WNV encephalitis (**Table 1**). No protective effect was observed in these or other published studies (17, 59, 78) with isotype control antibodies.

To characterize in greater detail the protective activity of the more potent of the two neutralizing MAbs, CR4354, infection studies with wild type and K136E escape variant WNV were repeated in highly susceptible three to four week-old NIH Swiss Webster mice (**Table 2**); this strain was selected because WNV morbidity and mortality shows a linear virus dose dependence (4). Consistent with the cell culture plaque morphology data, the lethality of wild type and K136E WNV-NY99ic was not different, with LD_{50} values of 0.28 and 0.24 PFU, respectively ($P > 0.8$). Sequencing of viral RNA from brains of mice infected with wild type or K136E variant WNV-NY99ic revealed no amino acid changes: WNV in the brain after infection with wild type and mutant viruses contained K and E residues at position 136, respectively (data not shown). To confirm the neutralization escape phenotype, mice were pre-treated with 50 μg (~100 times the minimum protective dose in C57BL/6 mice) of CR4354 MAb one day prior to infection with ~3 x 10^1 PFU of wild type or K136E variant WNV-NY99ic. CR4354 protected 94% (15 of 16) of mice from WNV-NY99ic challenge, whereas only 19% (3 of 16) of mice challenged with K136E-NY99ic survived despite CR4354 prophylaxis. In contrast, Hu-

E16 protected nearly all mice after infection with either wild type or K136E-NY99ic. Collectively, these data confirm the highly protective activity of CR4354 in vivo and the CR4354 escape phenotype of the K136E mutation.

DISCUSSION

Previous studies suggested that antibodies that map to the DIII-lr epitope do not account for the majority of neutralizing activity in serum from human patients or horses (60, 70). In this study, we characterized the functional properties of two neutralizing human MAbs, CR4348 and CR4354 that recognize distinct epitopes. Both MAbs inhibit WNV infection in vitro and in vivo but did not recognize closely or distantly related flaviviruses, including SLEV or DENV (M. Throsby, M. Vogt and M. Diamond, unpublished results). Biochemical studies demonstrate that these MAbs have similar but not identical profiles: they bind to conformationally sensitive epitopes on E proteins displayed on virions or SVP in a pH-sensitive manner but recognize recombinant E protein in solution or displayed on the surface of yeast poorly, if at all. Functional experiments suggest that both human MAbs neutralize infection primarily at a post-attachment stage in the viral lifecycle, specifically through inhibition of viral fusion with the endosomal membrane.

The CR4354 recognition site was established by neutralization escape and reverse genetic experiments and localized to the DI-DII hinge interface at residue K136. This hinge is highly flexible, as the angle between DI and DII on DENV E rotates 27º during the transition from the immature to mature state after furin-mediated cleavage of prM (87). The hinge then rotates back 30º during the pre- to post-fusion domain rearrangement (7, 49). Two X-ray crystal structures of similarly prepared WNV E protein also have distinct DI-DII hinge angles, differing by 5º (30, 58). This inherent flexibility of the DI-DII hinge could explain the lack of CR4354 binding to soluble and yeast surface displayed E, as these recombinant E proteins may not display the native hinge

that is found on virions or SVP. Indeed, exposure of SVP to acidic conditions that alter the DI-DII hinge angle significantly reduced binding of CR4354.

Although the characteristics of CR4354 appear relatively unique among anti-WNV MAbs, several other anti-flavivirus neutralizing MAbs have been described that localize to this region (**Table 3**). The anti-WNV MAb that maps closest to this region is the mouse MAb E113, which binds a determinant along the DI-DII hinge interface at residues E49 and K208 (**see Fig 6C**). E113 is protective in vitro (EC_{50} ~0.25 μg/ml) and in vivo, but unlike CR4354, E113 binds recombinant and yeast-displayed E proteins efficiently (61). The importance of this epitope for antibody neutralization is reflected by the characterization of several DI-DII inhibitory anti-flavivirus MAbs. Notably, MAbs (503, NARMA3, and B2) that recognize the DI-DII hinge are strongly inhibitory against the closely related JEV, which is a member of the same serogroup as WNV (21, 27, 35, 52).

Neutralization escape experiments suggested T208 and H246 as recognition sites near the DII dimer interface for the CR4348 MAb. The importance of these residues for neutralization was confirmed using reverse genetic approaches by introducing these mutations into WNV RVP and the infectious cDNA clone. Although mutation of these two residues abolished neutralization they did not prevent CR4348 MAb binding in capture ELISA experiments, suggesting that other amino acids that were not revealed in our selection experiments contribute to the epitope. While T208 and H246 lie spatially far apart (~43Å) within the E monomer, likely beyond the footprint of the antibody paratope, they reside significantly closer (~19Å) across the dimer interface. Thus, CR4348 may recognize an epitope that is sensitive to the oligomeric state of the E protein. Consistent

with this, CR4348 does not stably bind WNV E on yeast or in the solid-phase when adsorbed at low temperature (4ºC). However, when WNV E was adsorbed to microtiter plates at 37ºC we reproducibly observed modest levels of binding. Furthermore, CR4348 binds poorly to SVP that have been exposed to mildly acidic solutions; a decrease in pH induces a structural rearrangement in flavivirus E proteins, resulting in dimer dissociation and trimer formation (7, 49).

The CR4348 MAb epitope is structurally and functionally unique among characterized anti-WNV MAbs. Although CR4348 recognizes residues at the DII dimer interface that are proximal to the E100 mouse anti-WNV E MAb (61), the two differ in several respects: E100 maps more distal from DI at residue H263, only modestly neutralizes WNV in cell culture (EC_{50} ~ 10 μg/ml), recognizes both soluble and yeast forms of E, and shows quite limited protective efficacy against lethal WNV infection in mice. However, a DII-dimer interface MAb with more similar properties has been described for the distantly related flavivirus, TBEV (**Table 4**). The A5 MAb (26) maps to residue E207 along the dimer interface (45), is strongly neutralizing in culture (26), and partially blocks TBEV fusion in the pyrene excimer liposome fusion assay (74). Moreover, binding of at least some cross-reactive neutralizing flavivirus MAbs (e.g., 4G2 and 6B6C-1) that map to the fusion peptide in DII are also affected by mutation of residues (E231) along a dimer interface (10). One speculation as to why so few MAbs with these functional properties have been described is that they may not be identified with screens or immunization protocols using recombinant E proteins.

One limitation to mapping analyses by neutralization escape, which may be important for MAbs that show loss of function with mutations in a flexible hinge, is the

inability to exclude a distal binding site that is modulated allosterically by the hinge. A lack of binding to yeast or soluble E could reflect that these recombinant forms of E are truncated at amino acid 415 and lack the C-terminal stem-anchor and transmembrane regions. The lack of identification of an escape mutant in the highly conserved C-terminal regions could be due to poor viability of these variants. Unfortunately, because these MAbs bind poorly to recombinant E proteins, co-crystallography (44, 57), nuclear magnetic resonance (82), or saturation mutagenesis (23, 24, 46) approaches to identify the structural epitope are not possible. Instead, cryo-electron microscopy studies with Fab-virion complexes (31, 44) are planned to confirm the location of the epitope on the virion. Such experiments will be especially important for mAbs like CR4348, which shows a loss of neutralization but not binding with mutations at the DII dimer interface.

We previously described E16, a strongly neutralizing WNV-specific mouse MAb that maps to DIII-lr of the E protein (59) and inhibits infection by blocking viral membrane fusion in endosomes (B. Thompson, B. Moesker, J. Wilschut, J. Smit, M. Diamond, and D. Fremont, submitted for publication). Although CR4348 and CR4354 also strongly neutralize infection and affect a post-attachment step in the viral life cycle, they likely inhibit WNV by a mechanism distinct from that of E16. CR4354 appeared to inhibit infection almost equivalently when it was added before or after attachment. In contrast, CR4348 and E16 showed somewhat enhanced neutralizing activity when added prior to attachment. Moreover, in the liposomal model system, E16 completely blocked fusion at different pH values ranging from 6.3 to 5.0, consistent with ELISA and surface plasmon resonance data showing pH-independent binding of E16 to SVP (B. Thompson et al, submitted). In contrast, CR4354 and CR4348 do not completely block low-pH-

induced fusion of the virus with liposomes, with some residual fusion activity observed, respectively. This residual fusogenic activity in the setting of saturating concentrations of CR4354 and CR4348 may be due to heterogeneity among the WNV virions. Indeed, we also observed a small resistant fraction of virions in the classical $PRNT_{50}$ analyses under conditions of antibody saturation, with ~10% and 20% of the virus remaining infectious in the presence of high concentrations of CR4354 and CR4348, respectively (**Fig 1A**). At present the molecular basis of the differences in residual infectivity or fusogenic activity of the two experimental systems under conditions of CR4354 and CR4348 excess remains uncertain. The residual non-neutralized fractions, however, are not likely related to the maturation stage of the virus (56), since mature and partially immature WNV RVP are neutralized equivalently by these MAbs. More detailed analysis of antibody-virion structures and the precise pH dependency of the inhibition are planned to further define the mechanisms of neutralization.

For WNV and other flaviviruses, passive immunotherapy has been shown to protect small animals against lethal infection even when administered several days after infection (22, 59, 68). As recent studies suggest that resistance to monotherapy with the E16 MAb can occur in vivo (84), the use of combinations of neutralizing MAbs that recognize distinct epitopes may be advantageous as has been demonstrated for a therapeutic antibody cocktail against the rabies virus (2). The characterization of potently neutralizing MAbs like CR4348 and CR4354 that map to distinct regions and inhibit by different mechanisms suggests that this may be feasible.

TABLE 1. Effect of MAb pretreatment on survival of C57BL/6 mice

MAb	Dose (μg)	# of survivors/ total # of mice	% Survival	*P* value[a]
PBS		4/20	20	
CR4348	14	4/4	100	≤0.01
CR4348	4.2	5/5	100	≤0.01
CR4348	1.4	5/5	100	≤0.01
CR4348	0.42	3/5	60	0.13
CR4348	0.14	3/5	60	0.10
CR4348	0.042	2/5	40	0.31
CR4348	0.014	1/5	20	0.70
CR4354	14	5/5	100	≤0.01
CR4354	4.2	5/5	100	≤0.01
CR4354	1.4	5/5	100	≤0.01
CR4354	0.42	5/5	100	≤0.01
CR4354	0.14	4/5	80	0.018
CR4354	0.042	2/5	40	0.21
CR4354	0.014	1/5	20	0.57

[a] *P* values were determined using the log rank test and are compared to the PBS controls. C57BL/6 mice were pre-treated with the indicated dose of human MAb at day -1 by intraperitoneal injection. On day 0, mice were infected with WNV by subcutaneous route and monitored for survival.

TABLE 2. Effect of K136E substitution on MAb protection of NIH Swiss mice

MAb	NY99ic WNV	# of survivors/ total # of mice	% Survival	*P* value[a]
PBS	wild type	4/16	25	
Hu-E16	wild type	16/16	100	<0.0001
CR4354	wild type	15/16	94	<0.0001
PBS	K136E	1/16	6	
Hu-E16	K136E	15/16	94	<0.0001
CR4354	K136E	3/16	19	0.37

[a] *P* values were determined using the log rank test and compared to the PBS controls for each virus.

NIH Swiss mice were pre-treated with 50 μg of human MAb (Hu-E16 or CR4354) at day -1. On day 0, mice were infected with 3 x 10^1 PFU/mouse of WT-NY99ic or K136E-NY99ic virus and monitored for survival.

TABLE 3. Flavivirus MAbs that localize to the DI-DII Hinge Interface

Name of MAb	Virus	Epitope	Immunogen	Source	In vitro Neut.	In vivo Neut.	Ref.
CR4354	WNV	K136	Natural infection	Human	++	++	(78) and current paper
E113	WNV	E49, K280	Infectious virus + recombinant E protein	Mouse	+	+	(61)
503	JEV	S275, K136, I126	Infectious virus	Mouse	++	++	(34, 35, 52)
NARMA3	JEV	Q52	Infectious virus	Mouse	++	ND	(27, 37)
B2	JEV	I126	Vaccine + infectious virus	Chimpanzee	++	++	(21)
4B6C-2	MVEV	A126, R128, F274, S276, S277	Purified virus	Mouse	++	++	(28, 47)

++: greater than 90% inhibition via any in vitro assay, or capable of 90% protection in any lethal in vivo model

+: any significant in vitro neutralization or in vivo protective capacity

-: no significant in vitro neutralization or in vivo protective capacity

ND: not determined

TABLE 4. Flavivirus MAbs that localize to the DII Dimer Interface

Name of MAb	Virus	Epitope	Immunogen	Source	In vitro Neut.	In vivo Neut.	Ref.
CR4348	WNV	T208, H246	Natural infection	Human	++	++	(78) and current paper
E100	WNV	H263	Infectious virus + purified E	Mouse	+	+	(61)
A5	TBEV	E207	Solubilized virus	Mouse	+	ND	(26, 45)

++: greater than 90% inhibition via any in vitro assay, or capable of 90% protection in any lethal in vivo model

+: any significant in vitro neutralization or in vivo protective capacity

-: no significant in vitro neutralization or in vivo protective capacity

ND: not determined

FIGURE LEGENDS

Figure 1. **Neutralization and enhancement of human MAbs. A**. $PRNT_{50}$ assay. Hu-E16, CR4348, CR4354, and an isotype control MAb (DENV1-E50) were tested for neutralization activity by standard PRNT assay on BHK21-15 cells. Data shown are combined results of two independent experiments in triplicate. The data is normalized to data from from six control wells in each experiment with no MAb. **B-C.** RVP neutralization assay. Hu-E16, CR4348 and CR4354 were incubated with RVP prior to infection of Raji-DCSIGNR cells. RVP were prepared (**B**) normally or with the (**C**) T332K mutation in the E protein, which abrogates neutralization by virtually all all DIII-lr MAbs. Data shown are representative of three independent experiments in duplicate. Error bars represent standard deviation and lines represent curve fits generated by nonlinear regression analysis. **D**. Hu-E16, CR4348, and CR4354 were tested for their ability to enhance WNV RVP infection of human CD32 (Fc-γRIIA) expressing K562 cells. Data shown are representative of at least two experiments performed in triplicate. Error bars represent standard deviation.

Figure 2. Binding of human MAbs to recombinant protein, SVP, and virions. **A.** Yeast display assay. WNV E protein ectodomain (amino acids 1-415, left) or DIII alone (amino acids 296-415, right) was expressed on the surface of yeast, stained with CR4348 (*dotted*), CR4354 (*dashed*), Hu-E16 (*solid*), or irrelevant human IgG1 (*filled*) MAbs, and detected by flow cytometry. One representative experiment of three is shown. **B**. ELISA. The ectodomain (amino acids 1-415) of E protein was adsorbed to microtiter plates for one hour at 37ºC or overnight at 4ºC. After blocking, wells were incubated with the indicated MAbs and ELISA was performed as detailed in the Methods. Data shown

are representative of three experiments performed in triplicate with error bars representing standard deviation. **C**. Western blot. Recombinant E protein ectodomain (~39 kDa), SVP (glycosylated full length E protein, ~44 kDa), or WNV virus (unglycosylated full length E protein, ~43 kDa) were assessed for binding by indicated human MAbs to E or prM (~15 kDa) proteins. Samples were prepared in SDS sample buffer with or without heating to 95ºC and with or without 5% (v/v) β-mercaptoethanol (β-ME). **D**. A capture ELISA was used to detect binding of MAbs to WNV SVP (*left*) and virions (*right*). Microtiter plates were coated with murine E16, incubated with SVP or virus, and detected with the indicated human IgG1 MAbs. Dashed lines indicate the background of the assay with an isotype control MAb.

Figure 3. Effect of virus maturation state on neutralization by CR4348 and CR4354. CR4348 (*left panel*) and CR4354 (*right panel*) were incubated with MAbs prior to infection of Raji-DCSIGNR cells. RVP were prepared normally (mixture of mature, immature, and partially mature), in the presence of NH_4Cl (immature), or in cells over-expressing the furin protease (mature) to create virions of different maturation states. These were incubated with MAbs prior to infection of Raji-DCSIGNR cells. Data shown are combined results of three independent experiments in duplicate.

Figure 4. Mechanism of WNV neutralization by CR4348 and CR4354. A. Pre- and post-attachment inhibition assays. To determine whether the MAbs neutralize WNV infection after cellular attachment, Vero cells were pre-chilled to 4 ºC and 10^2 PFU of WNV was added to each well for one hour at 4 ºC. After extensive washing at 4ºC, the MAbs were added for one hour at 4ºC, and then the PRNT protocol was completed (*dashed lines*, *Post*). In comparison, a standard pre-incubation PRNT with all steps

performed at 4ºC is shown for reference. In this case virus and MAb are incubated together for one hour at 4°C, prior to addition to cells (*solid lines, Pre*). Data shown are representative of three experiments performed in duplicate with error bars representing standard deviation. **B**. pH sensitivity of MAb binding to SVP. The capture ELISA protocol was modified such that the pH was changed (6.0 or 7.4) for 30 minutes immediately before captured SVP were detected by indicated human IgG1 MAbs. One representative experiment of three performed in triplicate is shown, with error bars representing standard deviation. Dashed lines indicate the background of the assay with an isotype control MAb. **C-D**. Fusion of pyrene-labeled WNV with liposomes. **C**. Pyrene-labeled WNV was incubated with or without 50 nM of the indicated MAbs for one hour at room temperature prior to mixing with liposomes and acidification at pH 6.3. Fusion was measured on-line, as described in Materials and Methods. Representative fusion data of at least three independent experiments is shown. Curve *a*, no antibody; *b*, 50 nM isotype-matched control; *c*, 50 nM CR4348; *d*, 50 nM CR4354. **D.** Extent of WNV fusion at increasing concentrations of antibody. The extent of fusion was determined at 60 seconds upon acidification and is shown as a percentage of the control (no antibody, pH 6.3). White bars, isotype-matched control MAb; dark gray, CR4348; light gray, CR4354. Representative fusion data of at least three independent experiments is shown.

Figure 5. Characterization of neutralization escape mutants. A. PRNT assay performed in duplicate with virus after three passages under selection of either CR4348 (left) or CR4354 (right) on Vero cells. Reduced neutralization capacity was observed compared to Hu-E16. **B-C**. Confirmation of resistant phenotype with reverse genetic

RVP or infectious cDNA clone assays. Mutated (**B**) RVP and (**C**) infectious WNV were used to confirm that single amino acid substitutions of T208I (*left*), H246Y (*middle*), and K136E (*right*) in the E protein could reduce or eliminate neutralization by selecting MAb. In each series of experiments the reduced neutralizing capacity of the indicated human MAb is compared to Hu-E16, which maps to a distinct epitope on the DIII-lr. The data are combined data from two or three separate experiments performed in duplicate or triplicate. Error bars indicate standard deviation. Lines represent curve fits generated by non-linear regression analysis. **D**. A capture ELISA was used to detect binding of MAbs to mutated (K136E, T208I, and H246Y) WNV virions. Microtiter plates were coated with murine DIII-lr MAb, incubated with mutated virus, and detected with the indicated human IgG1 MAbs. Dashed lines indicate the background of the assay with an isotype control human MAb. The data are representative of three separate experiments performed in triplicate. Error bars indicate standard deviation.

Figure 6. Structural mapping of MAb epitopes. A. WNV E protein dimer with K136 (CR4354) colored in blue and T208 and H246 (CR4348) colored in magenta. **B.** Close-up view of the WNV E DII dimer interface with the CR4348 epitope highlighted in magenta. **C**. Surface display model of WNV E DI-DII hinge region with epitope recognition sites of CR4354 and the corresponding WNV residues of other anti-flavivirus MAbs (E113, 503, NARMA3, B2, and 4B6C-2) listed in Table 4. Note that residue 136 is labeled CR4354 but also is part of the 503 epitope and residue 126 is labeled B2 but also is part of the 503 and 4B6C-2 epitopes.

FIGURE 1

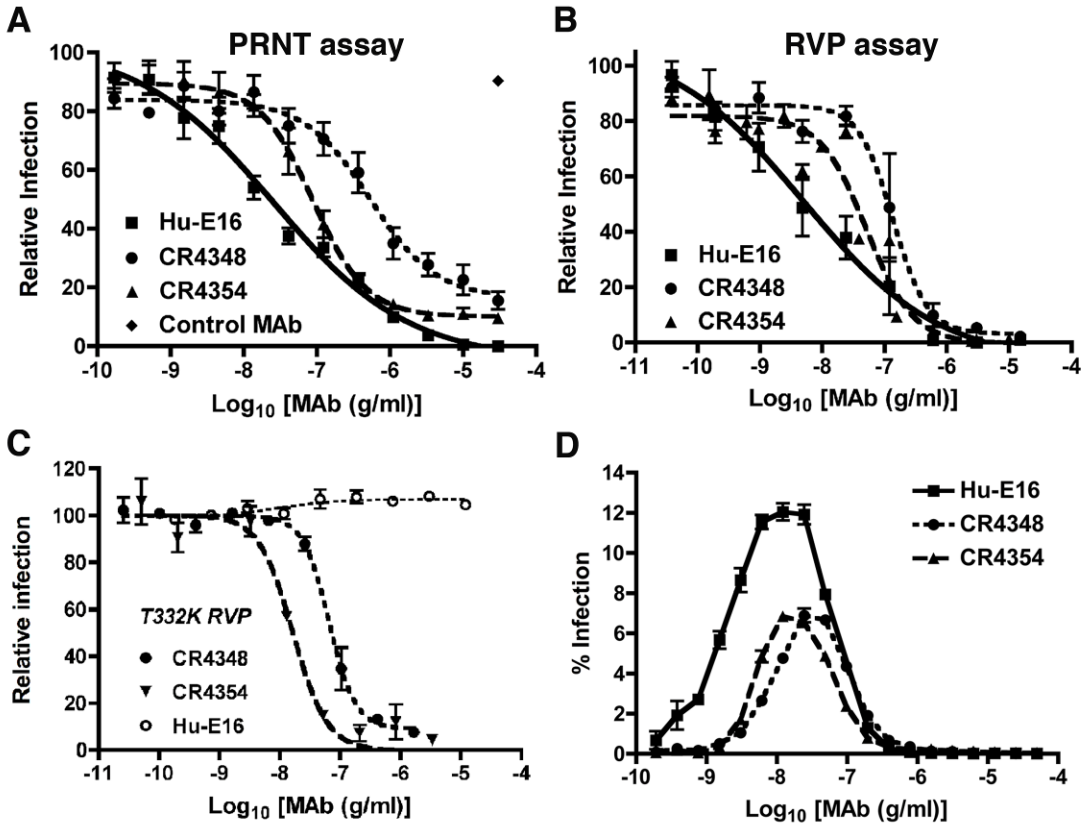

A
PRNT assay
Relative Infection
Hu-E16
CR4348
CR4354
Control MAb
Log10 [MAb (g/ml)]
B
RVP assay
Relative Infection
Hu-E16
CR4348
CR4354
Log10 [MAb (g/ml)]
C
Relative infection
T332K RVP
CR4348
CR4354
Hu-E16
Log10 [MAb (g/ml)]
D
% Infection
Hu-E16
CR4348
CR4354
Log10 [MAb (g/ml)]

FIGURE 2

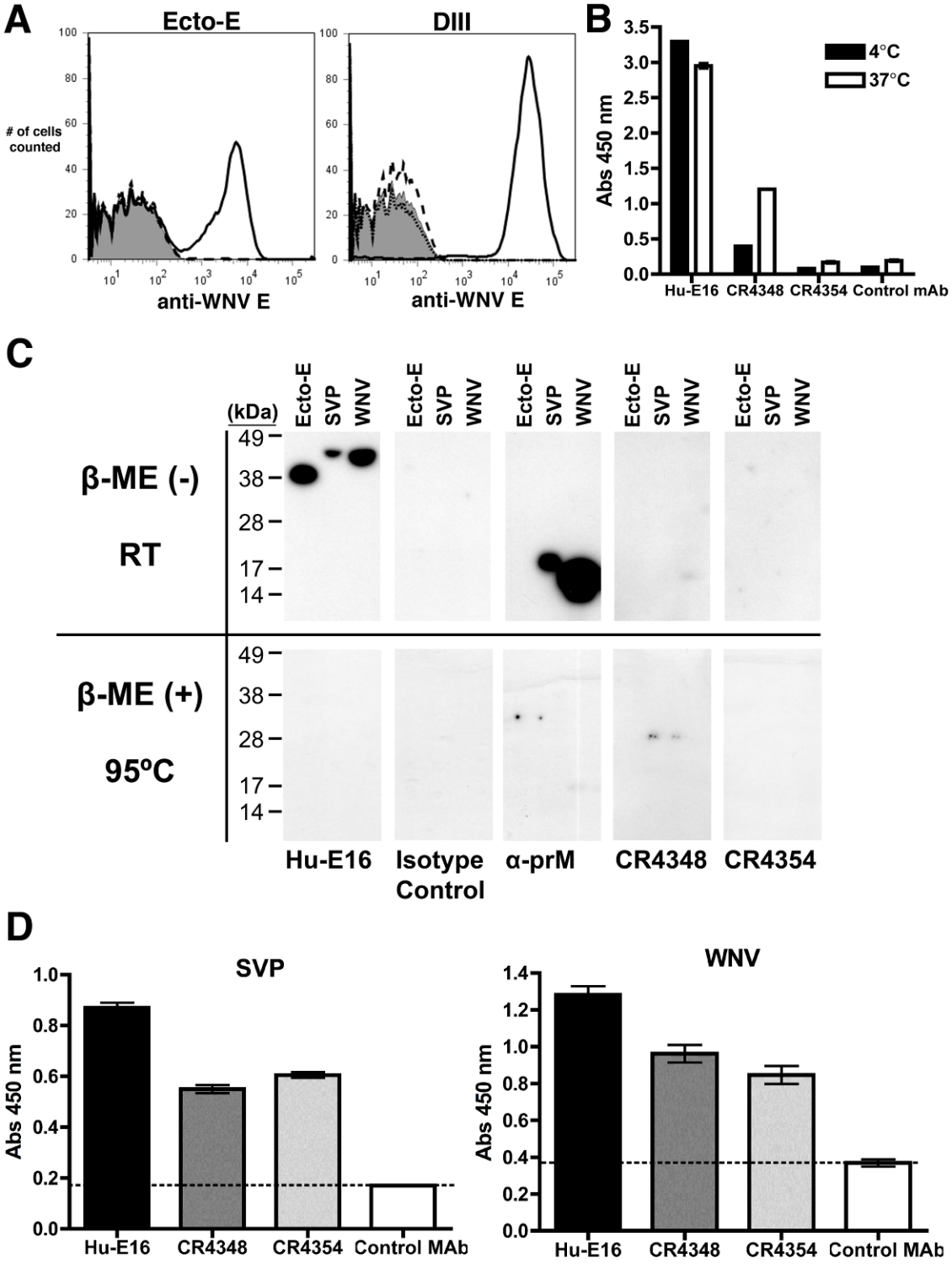

A
Ecto-E
DIII
# of cells counted
anti-WNV E
anti-WNV E
B
4°C
37°C
Abs 450 nm
Hu-E16
CR4348
CR4354
Control mAb
C
(kDa)
Ecto-E
SVP
WNV
β-ME (-)
RT
β-ME (+)
95ºC
49
38
28
17
14
Hu-E16
Isotype Control
α-prM
CR4348
CR4354
D
SVP
WNV
Abs 450 nm
Hu-E16
CR4348
CR4354
Control MAb

FIGURE 3

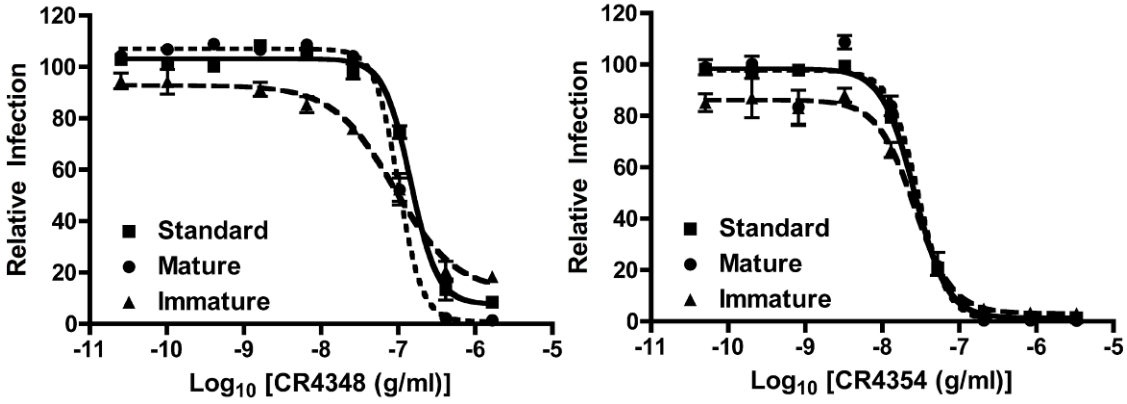

Relative Infection
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
Standard
Mature
Immature
Log10 [CR4348 (g/ml)]
Relative Infection
Standard
Mature
Immature
Log10 [CR4354 (g/ml)]

FIGURE 4

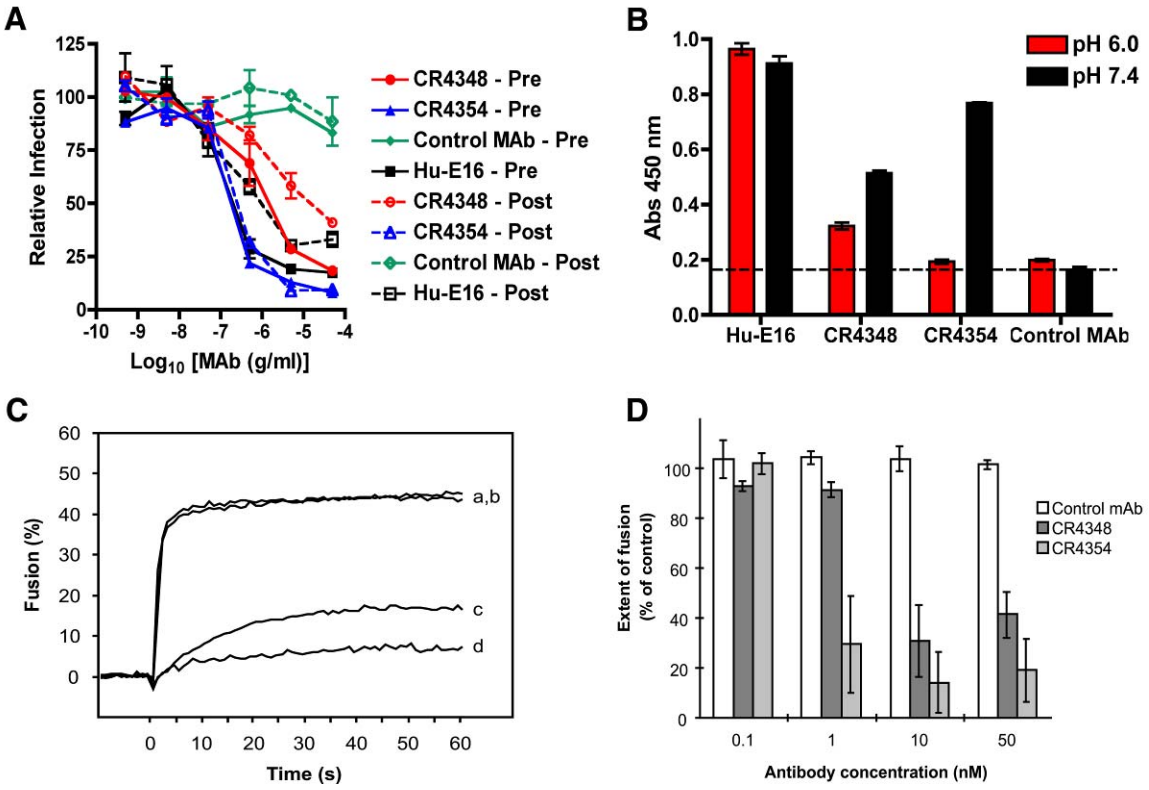
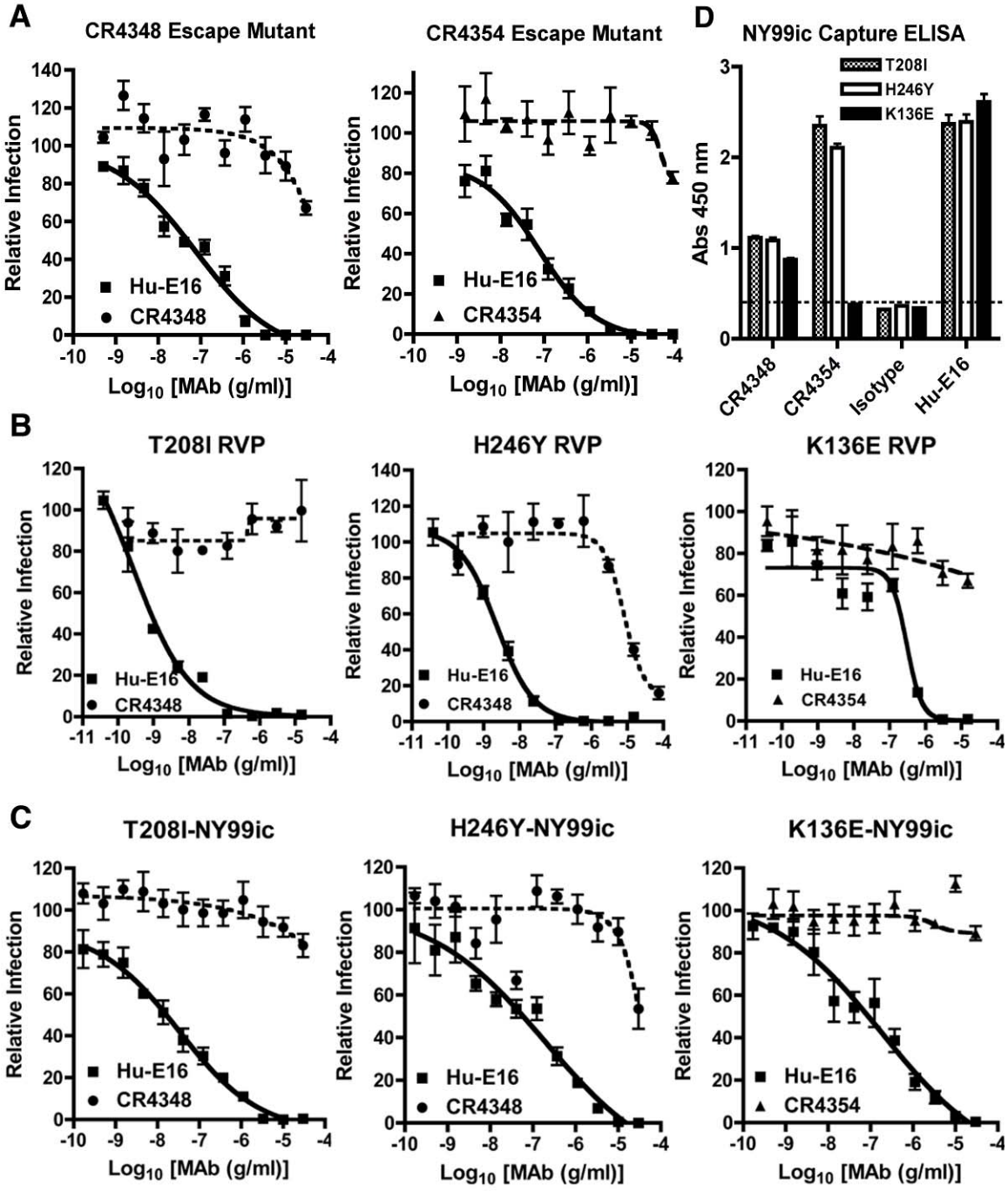

A
Relative Infection
125
100
75
50
25
0
-10 -9 -8 -7 -6 -5 -4
Log10 [MAb (g/ml)]
CR4348 - Pre
CR4354 - Pre
Control MAb - Pre
Hu-E16 - Pre
CR4348 - Post
CR4354 - Post
Control MAb - Post
Hu-E16 - Post
B
Abs 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
pH 6.0
pH 7.4
Hu-E16
CR4348
CR4354
Control MAb
C
Fusion (%)
60
50
40
30
20
10
0
a,b
c
d
0 10 20 30 40 50 60
Time (s)
D
Extent of fusion (% of control)
100
80
60
40
20
0
Control mAb
CR4348
CR4354
0.1
1
10
50
Antibody concentration (nM)

FIGURE 5


A
CR4348 Escape Mutant
CR4354 Escape Mutant
D
NY99ic Capture ELISA
T208I
H246Y
K136E
Abs 450 nm
CR4348
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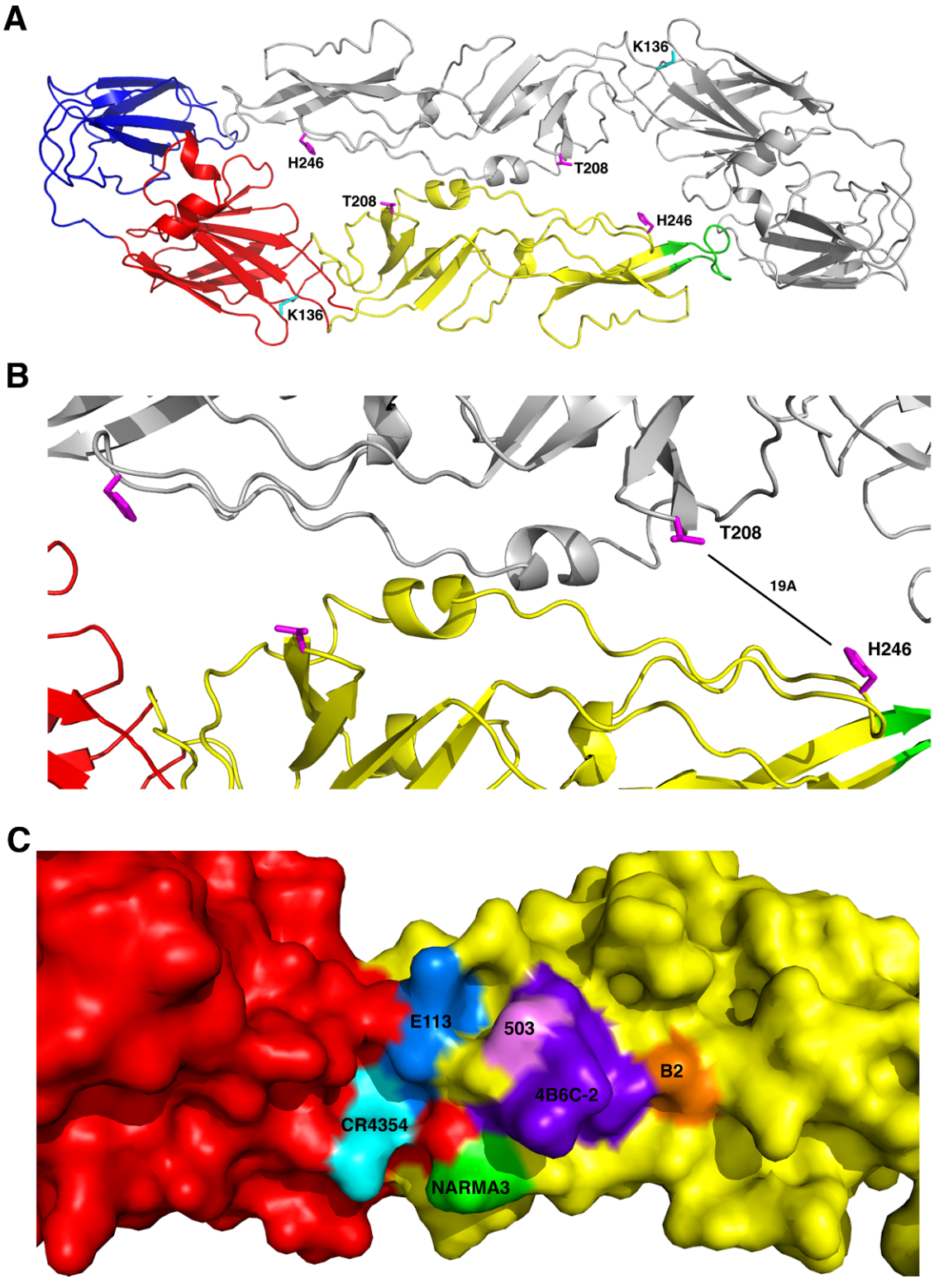
Isotype
Hu-E16
B
T208I RVP
H246Y RVP
K136E RVP
C
T208I-NY99ic
H246Y-NY99ic
K136E-NY99ic
Relative Infection
Log10 [MAb (g/ml)]
Hu-E16
CR4348
CR4354

FIGURE 6


A
K136
H246
T208
T208
H246
K136
B
T208
19A
H246
C
E113
503
B2
4B6C-2
CR4354
NARMA3

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Chapter III

Poorly Neutralizing Cross-Reactive Antibodies Against the Fusion Loop of West Nile Virus Envelope Protein Protect *in vivo* via Fc-γ Receptor and Complement-Dependent Effector Mechanisms

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INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted flavivirus that can infect and cause disease in humans and many other vertebrate animals. The flavivirus genus also contains other human pathogens of global relevance, including dengue (DENV), yellow fever, Japanese encephalitis, and tick borne encephalitis viruses. Most human WNV infections are asymptomatic, but about 20% of infected individuals experience a mild fever, and less than one percent develop severe neuroinvasive disease (67). Risk factors for symptomatic disease include an age of greater than 55 years, a compromised immune status, genetic variation in the OAS1 gene, and a CC5Δ32 genotype (17, 26, 40, 41). Although WNV first appeared in the Western hemisphere in 1999 in New York and spread rapidly through North America, surprisingly few human clinical infections have been reported in Central and South America despite the migration of avian hosts and appropriate vectors for transmission (35, 56).

WNV infection requires attachment to cell surface receptors, which remain poorly defined, endocytosis, and acid-catalyzed fusion of the virus within the late endosome. After translation of input strand RNA and viral replication, progeny virion assembly occurs within the endoplasmic reticulum (ER), with the capsid protein and genomic RNA associating with pre-membrane (prM) and envelope (E) proteins (42). Virus particles bud into the lumen of ER as immature virions in which E and prM proteins interact to form 60 heterotrimeric spikes with icosahedral symmetry (84). Transit of the immature virion through the mildly acidic compartments of the trans-Golgi network (TGN) triggers an extensive rearrangement on the virion surface. This low pH induced transition causes the E proteins on immature virions to lie flat as antiparallel dimers on the surface of the

virion (36), which in turn increases the susceptibility of prM to cleavage by a furin-like serine protease in the TGN (39, 82). Release of prM occurs in the neutral pH of the extracellular space (82). Mature flavivirus virions are relatively smooth particles that display 90 E protein dimers arranged in a herringbone pattern. While cleavage of prM is a required step in the viral lifecycle, it can be an inefficient process. Moreover, partially mature flavivirus virions containing some uncleaved prM also retain infectivity (29, 33, 51).

Studies in mice and other animals have established that humoral immunity is an essential component for protection against lethal WNV infection (57). B cells and secreted antibody were necessary for survival of mice after WNV inoculation (18, 19), and passive transfer of WNV-immune serum protected naive recipients from WNV challenge (22, 74). Moreover, pre-exposure prophylaxis and post-exposure therapy with WNV-specific monoclonal (MAb) or polyclonal antibodies conferred protection in both mice and hamsters (4, 6, 22, 48, 53, 71, 74, 75). The E protein of WNV is the principal target of neutralizing antibodies. Antibody neutralization occurs by blocking attachment to host cells, penetration of virions into cells, and the low pH-dependent fusion of the viral and host cell membranes (58). X-ray crystallographic analysis of several flavivirus E proteins has revealed a canonical structure with three domains (domain I (DI), domain II (DII) and domain III (DIII)). The generation and characterization of large panels of mouse and human MAbs against epitopes spanning the WNV E protein has enhanced our understanding of the antibody response to WNV. Although mouse MAbs that bind to all three domains of WNV E protein have been described, the most potently inhibitory MAbs recognize the lateral ridge epitope on DIII (DIII-LR) (2, 53, 64). In comparison,

the human anti-E repertoire appears more focused on a poorly neutralizing epitope on the fusion loop of DII (DII-FL) (54, 75).

In this study, we examined the contribution of poorly neutralizing antibodies to protection against WNV infection. In particular, a fusion-loop specific MAb (E28), which had little detectable inhibitory activity in cell culture, protected mice against lethal WNV infection. Protection required antibody effector function, as survival benefit was lost in mice lacking activating Fc-gamma receptors (FcγR) and the complement opsonin C1q or when an aglycosyl E28 variant that cannot engage FcγR and C1q was administered to wild type mice. In subsequent mechanistic studies, we show that E28 treatment decreased viral load in the serum early in the course of infection, an effect that required cells with phagocytic activity. Finally, we demonstrate that human and hamster polyclonal antibody responses after DENV infection that cross-react with WNV are generally poorly neutralizing and skewed to the DII-FL epitope, and that passive transfer of IgG purified from DENV-immune hamsters also protects mice from lethal WNV infection. Our study establishes the functional significance of immunodominant poorly neutralizing antibodies in the polyclonal human anti-flavivirus response and highlights the limitations of current neutralization assays, which cannot account for the protective effects conferred by these antibodies *in vivo*.

MATERIALS AND METHODS

Antibodies. All MAb and polyclonal antibodies used in this study were purified by protein A or protein G affinity chromatography. Anti-WNV mouse MAbs E24, E28, E34, and E53 have been described previously (53, 55). Mouse isotype controls recognized dengue virus envelope (E) (DENV-2 E70, IgG1) (70) and precursor-membrane (prM) (2H2, IgG2a) (25) glycoproteins and do not cross-react to WNV. Chimeric versions of MAb E28 (Ch-E28 and Ch-E28 N297Q) were generated by cloning the variable (V_H and V_L) regions of murine MAb E28 upstream of human IgG1 constant regions and performing site-directed mutagenesis as described previously for chimeric versions of MAb E16 (53). The negative control chimeric mouse-human IgG1 MAb (Ch-4420) was specific for fluorescein isothiocyanate (FITC) (3). PK136, a hybridoma producing mouse anti-mouse-NK1.1 MAb (IgG2a), was the gift of W. Yokoyama (St Louis, MO). RB6.8C5, a hybridoma producing rat anti-mouse-Gr-1 MAb (IgG2b), was the gift of E. Unanue (St Louis, MO). A negative control rat IgG was purchased (Jackson ImmunoResearch). Hamster IgG was isolated from the serum of naïve or DENV-2 infected golden Syrian hamsters (Harlan). Human serum was the gift of R. Akkina and S. Halstead (Pediatric Dengue Vaccine Initiative) and A. de Silva (Chapel Hill, NC). Serum was collected from individuals who had experienced multiple infections in the past with more than one serotype of DENV as judged by clinical history and PRNT titer analysis. The sera had the following reciprocal $PRNT_{50}$ values against DENV-1, DENV-2, DENV-3, and DENV-4, respectively: #1 (640, 611, 213, and 319); #2 (371, 320, 288, and >1,280); #3 (>1,280, 300, 99, and 266); #4 (>1,280, >1,280, >1,280, and 285); #5 (589, >1,280; 717, and 82); and #6 (>1,280, >1,280, >1,280, and 187).

The following antibodies against mouse antigens were conjugated to fluorescent markers and used in flow cytometry experiments: anti-CD11b-Alexa Fluor 647 (AF647) (M1/70), anti-CD45-peridinin chlorophyll protein (PerCP) (30-F11), anti-Ly6C-phycoerythrin (PE) (AL-21), and anti-Ly6G-PerCP-Cy5.5 (1A8). The conjugated antibodies listed above were purchased from BD Biosciences. Anti-CD3ε-AF647 (145-2C11) was purchased from BioLegend, anti-NKp46-PE (29A1.4) was purchased from eBioscience, and anti-human IgG-AF647 was purchased from Invitrogen.

Virus stocks. The WNV strain (3000.0259) was isolated in New York in 2000 and described previously (20). The virus was passaged twice in C6/36 *Aedes albopictus* cells to generate a stock virus that was used in all *in vivo* experiments. *In vitro* studies utilized a virus stock that was passaged one additional time in C6/36 or Vero cells. An *in vivo* derived stock of WNV was generated from plasma three days after infection of AG129 mice as described below. WNV subviral particles (SVP) were generated as described previously (80) by transiently transfecting a pcDNA3.1 plasmid encoding the prM and E proteins into 293T cells and harvesting cell culture supernatant after 48 hours. WNV reporter virus particles (RVP) that differ with respect to the extent of prM cleavage were generated as described previously (51).

Mouse experiments. Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. All wild type C57BL/6J mice were purchased from a commercial source (Jackson Laboratories). Congenic *C1q*$^{-/-}$ mice were originally obtained from M. Botto and G. Stahl (Imperial College, London and Beth Israel Deaconess Medical Center, Boston, MA). Congenic *FcγRI*$^{-/-}$ mice were originally obtained from S. Verbeek (Leiden

University Medical Center, Leiden, The Netherlands). Congenic common gamma chain-deficient (*FcγR*$^{-/-}$), which lack FcγRI, FcγRIII, and FcγRIV, and *FcγRIII*$^{-/-}$ mice were purchased commercially (Taconic). The *C1q*$^{-/-}$ x *FcγRI*$^{-/-}$, *C1q*$^{-/-}$ x *FcγR*$^{-/-}$ and *C1q*$^{-/-}$ x *FcγRIII*$^{-/-}$ mice were generated by crossing the individual knockout mice. All mice were bred and maintained in pathogen-free barrier facilities. Purified antibodies were administered to mice diluted in endotoxin-free PBS (HyClone Laboratories) by an intraperitoneal route. WNV was diluted in Hank's balanced salt solution (Mediatech, Inc.) containing 1% heat-inactivated fetal bovine serum (FBS) (Omega Scientific) and administered by subcutaneous injection in the footpad after anesthetization with xylazine and ketamine. For NK cell depletion, mice were administered the PK136 anti-NK1.1 antibody (100 μg) two days before and after infection with WNV. For neutrophil and inflammatory monocyte depletion, mice were given the RB6.8C5 anti-Gr-1 antibody (250 μg) one day before infection with WNV.

To isolate serum, blood was collected in Serum Gel Z tubes (Sarstedt), allowed to clot on ice for 30 minutes, centrifuged, and the liquid phase aliquoted and stored at -80ºC. Viral RNA was isolated from serum using the QIAamp Viral RNA Mini Kit (QIAGEN), stored at -80ºC, and quantified utilizing TaqMan quantitative reverse transcriptase qRT-PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems) as described previously (18). For plasma isolation, blood was collected into chilled ethylenediaminetetraacetic acid (EDTA) coated tubes (Becton Dickinson) on ice, centrifuged at 4°C, and the liquid phase aliquoted and stored at -80ºC. For tissue harvesting, mice were anesthetized with xylazine and ketamine and perfused extensively with PBS prior to organ dissection. Tissues were placed on dry ice, weighed, and stored

at -80ºC. Tissues were subsequently homogenized in PBS and virus was titrated by plaque assay on BHK21-15 cells.

Flow cytometry. For preparation of splenocytes as single cell suspensions, spleens were manually homogenized on 40 μm cell strainers into RPMI medium (Sigma). For preparation of peripheral blood leukocytes (PBL), blood was collected into EDTA-coated tubes, and erythrocytes were lysed by diluting whole blood in ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for five minutes. Splenocytes or PBL were washed thrice in PBS with 2% FBS prior to staining with cell type-specific conjugated MAbs for 30 minutes on ice. Cells were washed thrice and then analyzed on a FACSCalibur flow cytometer (BD Biosciences) using FlowJo software (Treestar).

Liposomes. Clodronate liposomes were prepared according to published methods (60, 79). Briefly, 8 mg of cholesterol and 86 mg of phosphatidyl choline (Sigma-Aldrich) were dissolved in chloroform. Excess chloroform was removed under vacuum. Clodronic acid disodium salt (Cl_2MDP, Sigma-Aldrich) was dissolved in de-ionized water and the pH adjusted to 7.3 with 5 N NaOH. The dry lipid pellet was resuspended in either 2.5 g of Cl_2MDP in 10 ml of water for macrophage-depleting liposomes or PBS for control liposomes. Liposomes were washed with PBS by ultracentrifugation (Beckman L-80 ultracentrifuge with 70.1 Ti rotor at 22,000 x g for 30 min) and resuspended in a final volume of 4 ml of PBS. To confirm that clodronate-containing lipsomes effectively depleted macrophages, C57BL/6 *RAG1*$^{-/-}$ mice were injected intravenously with 250 μL of liposome suspension and splenocytes were analyzed by flow cytometry 24 hours later for expression of CD11b and CD11c markers. For experiments in wild type mice,

liposomes were administered intravenously one day prior to and one day after WNV infection.

***Neutralization assays*. (i) PRNT.** Classical plaque reduction neutralization tests (PRNT) were performed as described previously on BHK21-15 cells after infection with 50 to 125 PFU of WNV (80), depending on the assay. EC_{50} values were generated by nonlinear regression analysis (GraphPad Software). **(ii) RVP.** Neutralization assays with WNV reporter virus particles (RVP) expressing GFP were performed with Raji-DCSIGN-R cells as described previously (59). **(iii) Multi-step growth curve.** WNV was mixed with individual MAbs (30 μg/ml) in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS for one hour at 37ºC, then added to a monolayer of BHK21-15 cells at a MOI of 0.001 and incubated for specified times at 37ºC. Supernatants were collected at the indicated times, aliquotted, stored at -80ºC, and quantified by plaque assay on BHK21-15 cells. **(iv) Raji-DCSIGN-R cells and infectious virus.** WNV was mixed with individual MAbs (30 μg/ml) in RPMI-1640 with 5% FBS for one hour at 37ºC and added to Raji-DCSIGN-R cells (MOI of 0.001) and incubated for 28 hours at 37ºC. Cells were fixed with 4% paraformadehyde, permeabilized with saponin (0.1% w/v), stained for WNV antigen (1^o Ab: Ch-E16; 2^o Ab: goat anti-human IgG-AF647) and processed on a FACSArray flow cytometer (BD Biosciences) using FlowJo software (Treestar).

***SVP and protein ELISA*.** Nunc MaxiSorp polystyrene 96-well plates were coated overnight at 4ºC with E24 MAb (10 μg/ml) in a pH 9.3 carbonate buffer. Plates were washed thrice in ELISA wash buffer (PBS with 0.02% Tween 20) and blocked for one hour at 37ºC with ELISA block buffer (PBS, 2% bovine serum albumin, and 0.02%

Tween 20). WNV SVP were captured for one hour at room temperature. Subsequently, plates were rinsed five times in wash buffer and then incubated with anti-WNV or flavivirus monoclonal or polyclonal antibodies (human or hamster) or controls (human IgG1 anti-FITC or naïve hamster antibodies) in duplicate for one hour at room temperature. Plates were washed five times and then incubated with biotinylated goat anti-human or hamster IgG (1:1000 dilution; Jackson ImmunoResearch) for one hour at room temperature in blocking buffer. After further rinsing, plates were incubated with 2 μg/ml of horseradish peroxidase conjugated streptavidin (Vector Laboratories) for 30 minutes at room temperature. Plates were washed six times and developed with tetramethylbenzidine (TMB) substrate (Dako). The reaction was stopped with 2N H_2SO_4 and emission (450 nm) was read on a TriStar microplate reader (Berthold Technologies). For quantification of the epitope specificity of anti-WNV E hamster IgG and human serum, plates were coated with 10 μg/ml of recombinant WNV E proteins produced in *E. coli* (wild type, W101R mutant, or E-quadruple mutant (T76R, M77E, W101R, and L107R)) as described previously (54). The E-quadruple mutant was the gift of C. Nelson and D. Fremont (St. Louis, MO). Equivalent site density was confirmed by measuring reactivity with E24 or Ch-E16 MAb, both of which recognize a distinct epitope on DIII-LR. Endpoint titers were defined as three standard deviations above the background optical density at 450 nm as determined by regression analysis using GraphPad Prism (GraphPad Software).

Western blotting. Individual preparations of WNV (10^6 PFU) were inactivated in 0.1% NP-40 detergent at 55ºC for 15 minutes. Subsequently, 4X lithium dodecyl sulfate sample buffer (Invitrogen) was added, and samples were heated to 95ºC for ten minutes,

centrifuged briefly, and loaded onto a Nu-PAGE 10% Bis-Tris gel (Invitrogen). After electrophoresis, the gel was rinsed in ddH_2O and protein was transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). Membranes were washed for ten minutes in wash buffer (PBS with 0.05% Tween 20), incubated overnight at 4ºC in blocking buffer (5% dry milk in wash buffer), and with human anti-E Ch-E16 MAb or polyclonal rabbit anti-M (Imgenex) at 1 μg/ml in blocking buffer for two hours. Following five rinses in wash buffer, membranes were incubated with horseradish-peroxidase conjugated goat anti-human IgG (Sigma) or anti-rabbit IgG (Thermo Scientific) diluted 1:2000 in blocking buffer for one hour. After five additional rinses, the blots were developed with Amersham ECL reagent (GE Healthcare) and visualized after exposure to x-ray film.

***Statistical analysis*.** Survival studies were analyzed using the log rank test. Comparisons of viral titers utilized either the Mann-Whitney non-parametric t test or one-way ANOVA as indicated. A paired student's t test was used for comparisons of hamster IgG and human serum titers against different recombinant E proteins. Neutralization curves were compared using the F test.

RESULTS

Poorly neutralizing MAbs protect mice from WNV infection. While the plaque reduction neutralization test (PRNT) and analogous assays are viewed as a standard measure of inhibitory activity of antibodies against many viruses, an imperfect correlation has been observed for DENV between the degree of neutralization *in vitro* and protection *in vivo* (9). To explore this concept further, we assessed the protective activity *in vivo* of several previously characterized murine MAbs against the WNV E glycoprotein and their corresponding neutralizing capacity as judged by the PRNT assay on BHK21-15 cells. In the absence of passively transferred antibody, as seen previously (22), administration of 10^2 PFU of insect cell-derived WNV via a subcutaneous route to 4 to 5 week-old wild type C57BL/6 mice resulted in a 14% survival rate (**Table 1**). While prophylaxis one day prior to infection with 40 μg of two strongly neutralizing domain III-lateral ridge-specific (DIII-LR) MAbs (E24 (IgG2a) and E34 (IgG1), $PRNT_{50}$: 4.0 and 80 ng/ml, respectively) provided strong protection (≥ 90% survival, $P < 0.0001$), passive transfer of poorly- (E53 (IgG2a)) and non- (E28 (IgG1)) neutralizing domain II-fusion loop-specific (DII-FL) MAbs still provided significant protection (43%, $P = 0.003$ and 60%, $P < 0.0001$ respectively), albeit it at lower levels (**Table 1; Fig 1A**). Thus, the capacity for anti-WNV MAbs to protect *in vivo* does not correlate perfectly with results from the PRNT assay in cell culture.

In vitro characterization of poorly neutralizing MAbs. The inability of the PRNT assay to predict MAb protection *in vivo* warranted further investigation. Previous studies established that the relative maturity of WNV virions as reflected by the degree of cleavage of prM protein affects the neutralizing activity of MAbs that recognize some (e.g., DII-FL) but not other (DIII-LR) epitopes (51). As seen previously (51), E53

neutralized infection in Raji-DCSIGN-R cells of mature WNV reporter virus particles (RVP) produced in a cell line over-expressing furin to a lesser extent than those generated in the parent cells, which produce a mixture of mature and partially immature virions. In comparison, E24 neutralized both types of RVP equivalently (data not shown). In contrast to the classical PRNT assay with BHK21-15 cells and similar to results with E53, E28 consistently showed a low but measurable neutralization (~30%) of mature RVP in Raji-DCSIGN-R cells (**Fig 1B**). The modest ability of E28 to neutralize WNV RVP was not a cell type-specific effect, as E28 (30 μg/ml) failed to neutralize infection of Raji-DC-SIGNR cells with C6/36-derived fully infectious WNV, and in fact enhanced infection compared to controls (**Fig 1C**).

To assess whether the maturation state of WNV also affected neutralization in the PRNT assay, we propagated WNV in Vero cells, which produce virions that are more mature relative to those derived from C6/36 insect cells (**Fig 1D** and (24)). Similar to data with insect cell-propagated WNV, DIII-LR-specific MAbs E24 and E34 completely neutralized Vero cell-derived WNV with similar $PRNT_{50}$ values, whereas E28 showed no appreciable inhibitory activity across a wide dose range (**Fig 1E**). Again, E53 showed partial neutralizing activity of Vero cell-derived WNV achieving ~50% overall inhibition, although its potency was shifted 500-fold ($P < 0.0001$) to a requirement for higher concentrations of MAb. Thus, E28 lacked neutralizing activity of insect or mammalian cell-generated WNV on BHK21-15 cells, whereas a decreased state of maturity of WNV RVP was associated with some inhibitory activity in Raji-DCSIGN-R cells.

Since the source of WNV affected the level of neutralization observed in different cell types, we assessed the capacity of MAbs to inhibit WNV infection with virus produced *in vivo*. Because the recovery of infectious extracellular WNV from plasma of wild type mice is challenging due to limited viremia, we infected highly susceptible *AG129* mice, which lack receptors required for type I and type II interferon signaling. Plasma-derived WNV was not neutralized by E28 at any concentration tested (**Fig 1F**), results that may be explained by the completely mature phenotype of WNV in plasma, as uncleaved prM was not observed by Western blot (**Fig 1D**). Of note, E24 was 600-fold less potent ($P < 0.0001$) at neutralizing infection of plasma-derived WNV ($PRNT_{50}$ = 2.4 μg/ml) than insect-derived WNV ($PRNT_{50}$ = 4.0 ng/ml), which could be due to the enhanced E protein content of plasma-derived WNV that was observed at equivalent PFU compared to WNV preparations from C6/36 and Vero cells (**Fig 1D**) or to the existence of a neutralization escape variant. Finally, higher concentrations of E28 paradoxically augmented infection of plasma WNV preparations. This could be due to MAb-induced virus aggregation, as BHK21-15 cells lack expression of FcγR, precluding the possibility of "classical" antibody-dependent enhancement of infection (30). Collectively, these results suggest that the PRNT assay on BHK21-15 cells does not always predict the protective activity of WNV in mice even when accounting for different sources of virus, including those derived *in vivo*.

To further characterize the intrinsic neutralization capacity of anti-WNV MAbs on BHK21-15 cells, we performed multi-step growth curve analysis. While the addition of E24 (30 μg/ml) prior to infection resulted in complete neutralization, equivalent concentrations of E28 provided no reduction in viral titer compared to the isotype

antibody or medium controls (**Fig 1G**). Despite conferring protection in mice in passive transfer studies, and in contrast to E24, E28 had limited inhibitory activity in cell culture that was maturation state and cell-type-dependent.

E28 MAb prophylaxis reduces viral load in wild type mice. To gain further insight into how prophylaxis with the poorly neutralizing E28 MAb protects against WNV infection *in vivo*, passive transfer experiments were repeated, but mice were sacrificed and their organs collected for viral burden analysis at different time points. Mice receiving E28 MAb had lower levels of WNV in serum at days 1 and 2 post-infection (~3.4-fold, $P < 0.002$ and ~6.5-fold, $P < 0.0001$, respectively) compared to the PBS-treated animals as measured by quantitative RT-PCR of viral RNA (**Fig 2A**). Correspondingly, mice treated with E28 MAb had decreased (~100-fold, $P < 0.04$) levels of infectious WNV in the brain at day 7 after infection (**Fig 2B**). Although differences of WNV in the brain did not attain statistical difference at day 5 ($P > 0.2$), nearly half of the E28 MAb-treated mice had levels at or below the limit of detection of the assay, whereas all PBS-treated mice had measurable WNV titers. Thus, treatment with E28 MAb altered the course of WNV infection at an early stage, which impacted dissemination and replication at later phases of pathogenesis.

C1q and activating Fc-gamma receptors are required for in vivo protection by E28 MAb. Since E28 MAb had little neutralizing activity in cell culture assays yet protected against WNV infection and pathogenesis, we hypothesized that Fc-mediated effector functions of the MAb contributed to the observed *in vivo* effects. To test this, congenic mice deficient in the complement component C1q were administered MAbs one day prior to WNV infection. Older 8 to 12 week-old mice were used for these studies as

these immunodeficient strains are inherently more susceptible to WNV pathogenesis compared to wild type mice (14, 43, 53) and older C57BL/6 mice (up through 24 weeks) are more resistant to infection (12, 22). In mice lacking C1q, which initiates the antibody-dependent classical pathway of complement activation, 400 μg of E53 MAb prevented ($P = 0.02$) mortality caused by WNV infection (**Table 2A**). Whereas a lower dose (40 μg) of E28 MAb increased the survival time of *C1q*$^{-/-}$ mice by three days ($P < 0.02$), this dose of E53 and E28 failed to provide statistically significant protection against WNV mortality (**Table 2A** and **Fig 3A**). The partial, albeit limited protection by E28 in *C1q*$^{-/-}$ mice was somewhat surprising as this MAb is of the IgG1 isotype, which binds mouse C1q with low affinity (8). However, some mouse IgG1 MAbs bind complement better than others (23). Consistent with a functionally significant interaction, in vitro experiments showed that the addition of mouse C1q limited E28-mediated enhancement of infection in cells expressing FcγR (data not shown).

Mice lacking the common γ-chain of activating FcγR lack surface expression and signaling from FcγRI (CD64), FcγRIII (CD16), and FcγRIV (52). To assess the impact of FcγR-mediated effector functions on the protective capacity of poorly neutralizing MAbs, prophylaxis studies were repeated in mice lacking the common γ-chain (*FcγR*$^{-/-}$). Passive transfer of 40 μg of E28 and 400 μg of E53 protected *FcγR*$^{-/-}$ mice against lethal WNV infection (**Table 2B** and **Fig 3B**). Because we hypothesized that complement and FcγR-mediated effector functions might jointly contribute to the protective capacity of poorly neutralizing MAbs, we generated *C1q*$^{-/-}$x *FcγR*$^{-/-}$ mice. Notably, 400 μg doses of E28 and E53 MAbs did not prevent lethality in *C1q*$^{-/-}$x *FcγR*$^{-/-}$ mice, although robust protection was observed with strongly neutralizing DIII-LR MAbs (**Table 2C** and **Fig 3C**). A

requirement for FcγRIII for E28- and E53-mediated protection was inferred from passive transfer experiments with *C1q*$^{-/-}$x *FcγRIII*$^{-/-}$ mice as statistically significant protection also was lost (**Table 2D**). Collectively, these results suggest that Fc-mediated effector functions (C1q and FcγR) are required for the protective activity of poorly neutralizing anti-WNV antibodies.

To confirm independently that Fc effector functions of poorly neutralizing MAbs were required for protection *in vivo*, we engineered chimeric versions of E28 that retained or lost the ability to interact with C1q and FcγR. The variable (V_H and V_L) regions of mouse E28 were cloned upstream of the human IgG1 constant regions, and a chimeric mouse-human MAb (Ch-E28) was expressed as a wild type and aglycosyl variant; the latter contains a mutation (N297Q) in the heavy chain that eliminates binding to C1q and all FcγR (72). Wild type Ch-E28 and N297Q Ch-E28 bound to WNV virions equivalently in a capture ELISA (**Fig 4A**) yet still lacked neutralizing activity as judged by PRNT assay on BHK21-15 cells with C6/36-derived WNV (**Fig 4B**). While wild type Ch-E28 provided significant protection against mortality ($P = 0.03$) in 4 to 5 week-old wild type mice infected with WNV, N297Q Ch-E28 failed to do so (**Fig 4C**). Accordingly, treatment with Ch-E28 but not Ch-E28 N297Q was associated with reduced WNV levels in the brain at day 7 after infection (**Fig 4D**). These experiments confirm that Fc effector functions are required for *in vivo* protection by the poorly neutralizing MAb E28.

B, T, and NK cells are not required for E28 MAb-mediated protection. We hypothesized that E28 MAb could promote viral clearance directly through enhanced complement and FcγR-dependent uptake, as described previously with MAbs against WNV NS1 (14, 15), or that E28-dependent immune complex formation and uptake could

promote antigen presentation and adaptive immune responses. To address the latter possibility, passive transfer studies were repeated in *RAG1*$^{-/-}$ mice, which lack B and T cells; as these mice are highly vulnerable to lethal WNV infection (22, 83), a lower infecting dose of virus (1 PFU) was used and protection was assessed by a virologic rather than survival endpoint. Notably, *RAG1*$^{-/-}$ mice receiving E28 MAb showed lower levels ($\geq$ 5-fold, $P < 0.008$) of WNV in serum over time compared to the isotype control (**Fig 5**). While this experiment does not completely eliminate a possible role of B and T lymphocytes in contributing to E28-mediated protection in vivo, it establishes that these cells are not required for protection *in vivo*.

Our prophylaxis experiments with *C1q*$^{-/-}$ and *C1q*$^{-/-}$ x *FcγRIII*$^{-/-}$ mice suggested that some effector functions utilized by E28 for protection occur through FcγRIII-dependent pathways. Given that natural killer (NK) cells express high levels of FcγRIII and use this receptor for antibody-dependent cellular cytotoxicity (ADCC) in vivo (62), we hypothesized that NK cells might be essential for E28-mediated protection. To assess this, 4 to 5 week-old wild type mice were administered 100 μg of a murine IgG2a control MAb or PK136, an anti-NK1.1 MAb that depletes NK cells (73). At days 2 and 4 after treatment, mice given PK136 showed significant depletion of NK cells as expected (**Fig 6A**). However, depletion of NK cells had no impact on E28 MAb-mediated protection as judged by survival (**Fig 6B**) in mice treated with PK136 (100 μg) one day prior to WNV infection and again three days after infection. Thus, NK cells are not necessary for E28-mediated protection from WNV.

Phagocytic cells contribute to E28 MAb-mediated protection. As B, T, and NK cells were not required for protection, we hypothesized that E28 might opsonize virus for

phagocytosis by cells expressing FcγR and complement receptors. To test their contribution to E28-mediated protection after WNV infection, we non-selectively depleted phagocytes using clodronate liposomes (77). To confirm depletion, splenocytes were analyzed for expression of the myeloid cell markers CD11b and CD45 one day after liposome injection. As expected, treatment with clodronate-containing liposomes depleted $CD45^{int}CD11b^{hi}$ and $CD45^{hi}CD11b^{int}$ cells (**Fig 7A**), which corresponds to myeloid cells with phagocytic capacity. To determine whether phagocytes were necessary for E28-mediated protection, 8 to 12 week-old wild type mice were administered liposomes containing clodronate or PBS and 500 μg of E28 or isotype control MAb one day prior to infection with 1 PFU of WNV, and then given a second dose of liposomes on day 1 post-infection. The experimental design was altered from the initial survival studies because wild type mice treated with clodronate liposomes are more vulnerable to WNV infection (5, 60). Clodronate liposome treatment resulted in a loss of protection by E28 MAb as judged by elevated levels of WNV in serum at days one and two post-infection compared to mice given liposomes containing PBS (**Fig. 7B**). Thus, phagocytes are required for the early reduction in viremia mediated by E28.

Neutrophils and inflammatory monocytes are innate immune cells that express FcγR and complement receptors and could contribute to E28 MAb-mediated clearance. To determine if these cells were necessary mice were administered RB6.8C5 (250 μg), a rat anti-mouse Gr-1 MAb that depletes cells expressing Ly6C and Ly6G (**Fig 7C**), including neutrophils, inflammatory monocytes, suppressor monocytes, and plasmacytoid dendritic cells (21). RB6.8C5 or control antibody was given one day prior to infection and E28 (500 μg) treatment. Notably, E28 MAb treatment decreased serum viremia on

day two post-infection regardless of whether mice had received RB6.8C5 or control antibody (**Fig 7D**), indicating that Gr-1 expressing cells were not required for E28-mediated protective effects in vivo. Combined with the clodronate liposome data, these results suggest that phagocytic $CD11b^+$ macrophages may be the primary cell type responsible for much of the protective activity of E28 *in vivo*.

Poorly neutralizing, E28-like polyclonal antibodies protect mice from WNV infection. While the WNV epidemic spread rapidly across North America, few human clinical infections have been reported in Central and South America despite the presence of the virus in avian and mosquito hosts (28, 56). Although this could reflect reporting bias, we hypothesized that prior widespread exposure to DENV and other endemic flaviviruses could result in production of polyclonal DII-FL-specific antibodies that failed to neutralize WNV infection by conventional PRNT analysis but still protected humans *in vivo*. Indeed, DII-FL antibodies are immunodominant in humans after infection with several flaviviruses (16, 54, 69, 75). To test whether polyclonal antibodies had E28-like epitope specificity and functional activity and would behave similarly against WNV *in vivo*, golden Syrian hamsters were inoculated with DENV type 2 (DENV-2) and polyclonal IgG was isolated by affinity chromatography from serum that was collected four weeks after infection. Hamsters were used rather than mice because the latter have their antibody response skewed toward the DIII-LR epitope (54). Notably, DENV-2 immune hamster IgG bound more avidly to WNV virions than naïve IgG in a capture ELISA (**Fig 8A**). DENV-2 IgG failed to neutralize WNV infection by PRNT assay on BHK21-15 cells (**Fig 8B**, $PRNT_{50}$ < 1/15 serum dilution), although some inhibition of Raji-DCSIGN-R cell infection was detected with partially mature but not mature WNV

RVP (**Fig 8C**). Thus, polyclonal DENV immune hamster IgG has similar in vitro neutralization characteristics as MAb E28.

The vast majority of DII-FL-specific MAbs, including E28, lose binding to recombinant E protein that encodes a single W101R mutation (54). However, E53 retains binding to E-W101R but does not bind a recombinant E protein with three additional mutations within and proximal to the fusion loop (T76R, M77E, W101R, and L107R (E-quadruple mutant)). Polyclonal DENV-2 immune hamster IgG that cross-reacted with WNV E protein was skewed toward the DII-FL epitope (W101R: 83% ± 13% binding, $P < 0.005$; quadruple mutant: 90% ± 7.7% binding, $P < 0.002$) as determined in direct binding assays to wild type and mutant recombinant E protein (**Fig 8D**). As most of the DENV-2 immune hamster cross-reactive IgG recognized the DII-FL epitope and did not neutralize WNV infection in BHK21-15 cells, we tested it for protective activity *in vivo*. Similar to results with E28 MAb, passive transfer of DENV-2 immune hamster IgG one day prior to WNV infection significantly ($P = 0.03$) protected 4 to 5 week-old mice compared to naïve IgG (**Fig 8E**). Thus, poorly neutralizing cross-reactive polyclonal antibodies derived from heterologous flavivirus infection can mitigate WNV infection *in vivo*.

The cross-reactive antibody repertoire of serum from DENV immune humans is skewed toward the DII-FL epitope. Having demonstrated that polyclonal anti-DENV-2 hamster IgG was non-neutralizing yet protective against WNV infection in mice, we questioned whether similar trends would be observed with human serum from DENV-immune individuals. Serum with cross-reactivity to WNV was obtained from patients with a history of infection of at least two serotypes of DENV (S. Halstead and A. de

Silva, personal communication). Analogous to that seen with hamster IgG, polyvalent DENV immune human serum that cross-reacts with WNV E protein was skewed toward the DII-FL epitope (**Fig 9A**). Although variability was observed in the loss of binding to E-W101R (51% ± 42% binding, $P < 0.05$), loss of binding to E-quadruple mutant was more consistent (82% ± 14% binding, $P < 0.005$). Only one of the six sera tested neutralized WNV infection by >50% ($PRNT_{50}$ = 1/180 for sample #3). None of the remaining five human immune sera neutralized WNV by 50% at the lowest serum dilution tested (**Fig 9B**, $PRNT_{50}$ < 1/20 serum dilution), establishing that humans infected with DENV produce DII-FL antibodies that cross-react to WNV yet poorly neutralize infection. However, given the limited available quantities of human sera, we could not directly test for protective capacity in passive transfer experiments in mice.

DISCUSSION

A prior study suggested that DII-FL MAbs neutralize WNV poorly *in vitro*, yet still can protect mice from WNV infection (55). Here, we examined in greater detail the ability of two DII-FL MAbs, E53 and E28, to inhibit infection *in vitro* and *in vivo*. These DII-FL MAbs neutralized WNV differentially according to the cell type used and the maturity of the virus stock, with fully mature WNV more resistant to neutralization. The DII-FL MAbs protected wild type mice from lethal WNV infection, and prophylaxis with E28 MAb decreased WNV viremia at days one and two after infection and reduced infection in the brain at seven days after infection. The protective activity of DII-FL MAbs was essentially abolished in mice lacking both C1q and FcγR or in wild type mice treated with an aglycosyl MAb variant, establishing that Fc effector functions mediate *in vivo* protection of poorly neutralizing MAbs. While studies with deficient mice and depleting antibodies indicated that B, T, and NK cells were not necessary for this protection, phagocytes were required. Cross-reactive polyclonal antibodies from DENV immune hamsters and humans also were poorly neutralizing against WNV *in vitro* and directed against the DII-FL epitope. Nonetheless, and analogous to data with E28 MAb, these cross-reactive polyclonal antibodies conferred protection in passive transfer studies in mice.

Using multiple *in vitro* assays with WNV derived from different cell sources, we confirmed the poorly neutralizing capacity of DII-FL MAbs against WNV. While the PRNT assay on BHK21-15 or Vero cells remains the "gold standard" measure of antibody neutralization of flaviviruses (61), imperfect correlations between titers *in vitro* and protection *in vivo* have been observed (9). One hypothesis as to why poorly

neutralizing antibodies protect *in vivo* is that the PRNT assay, which is usually performed with C6/36-cell derived viral stocks, does not account for the inherent neutralizing activity of all classes of antibody against flaviviruses. To determine whether the source of the WNV stock affected MAb inhibitory activity by PRNT assay, we tested WNV prepared in mammalian Vero cells or *in vivo* from the plasma of mice. Plasma rather than serum-derived WNV was used to avoid maturation artifacts associated with *ex vivo* activation of clotting cascade serine proteases, which might adventitiously cleave prM. Vero cell and plasma derived WNV was essentially mature and resistant to DII-FL MAb neutralization by PRNT assay, consistent with studies showing DII-FL MAbs poorly bind or neutralize mature forms of WNV (13, 51).

Because a prior study suggested differential inhibitory activity of anti-WNV MAbs mapping to DI and DII using RVP and Raji-DCSIGN-R cells (55), we expanded our analysis to determine whether poor neutralizing activity of MAbs showed cell type-specific effects. E28 did not neutralize insect or mammalian cell-derived WNV in Raji-DCSIGN-R cells; indeed, it paradoxically enhanced infection through an unknown mechanism as these cells lack expression of activating Fc-γR. Therefore Raji-DCSIGN-R cells were not inherently better than BHK21-15 cells at revealing the neutralization potential of E28. However, when less mature forms of RVP were prepared, E28 showed modest inhibitory activity on Raji-DCSIGN-R cells, analogous to that described with E53 (51). Thus, the neutralization capacity of DII-FL MAbs was cell type and maturation state-dependent, and the PRNT assay provided an incomplete evaluation of the inherent inhibitory activity of these antibodies. This idea is important to consider, as the humoral

response to vaccines against WNV (1) and DENV (49) is evaluated primarily using the PRNT assay.

The results of the cell culture studies suggested that DII-FL MAbs, such as E28, were at most weakly neutralizing and possibly non-neutralizing depending on the source of virus and assay used for evaluation. Nonetheless, E28 and other poorly neutralizing DII-FL MAbs were protective in passive transfer experiments in mice (55). The ability of poorly neutralizing MAbs to protect animals against virus infection is not inherently novel, as it has been observed with flaviviruses (27, 34, 55), alphaviruses (10, 38, 46, 66), coronaviruses (50), reoviruses (76), and rhabdoviruses (37). These studies were observational, however, and the mechanism of protection in animals remained uncharacterized. Based on its *in vitro* properties, we selected E28 for an in-depth analysis of the mechanism of protection in mice. Studies with both strongly and poorly neutralizing antibodies against herpesviruses (81), flaviviruses (65), retroviruses (32, 47), and poxviruses (7) established that Fc effector functions were required for reducing viral burden and clinical morbidity. Analogously, our passive transfer experiments with $C1q^{-/-}$ x $Fc\gamma R^{-/-}$ mice or an aglycosyl E28 variant in wild type mice demonstrate the necessity of Fc effector functions for protection by weakly or non-neutralizing antibodies.

As the Fc portion of an antibody can engage complement and FcγRs, we repeated infections in $C1q^{-/-}$, $Fc\gamma R^{-/-}$, and $C1q^{-/-}$ x $Fc\gamma R^{-/-}$ mice to gain further mechanistic insight into protective mechanisms. While some protective capacity was lost in $C1q^{-/-}$ mice, E28 and E53 failed to protect $C1q^{-/-}$ x $Fc\gamma RIII^{-/-}$ mice, implicating this FcγR as a key component of the survival phenotype conferred by poorly neutralizing MAbs. How do Fc effector functions enhance the protective potential of weakly neutralizing antibodies? As

a previous study had demonstrated that C5 was not required for protective antibody effects on WNV (44), virion lysis appeared an unlikely mechanism. However, DII-FL MAbs may become neutralizing in the presence of C1q, as it reduces the stoichiometric threshold required for antibody neutralization (45). Further mechanistic studies were aimed at identifying the cell type(s) *in vivo* that conferred the protective activity. As E28 still decreased viremia in WNV infected *RAG1*$^{-/-}$ mice, it seems unlikely that immune complex uptake, enhanced antigen presentation, and priming of the adaptive B and T cell responses explained the protective effects in vivo. The experiments in *RAG1*$^{-/-}$ mice were supported by prophylaxis studies with E53 showing no change in the kinetics of $CD4^+$ or $CD8^+$ T cell activation or quality of the neutralizing antibody response after WNV infection in wild type mice (M. Vogt and M. Diamond, unpublished studies).

Although NK cells appear unnecessary for protection against primary WNV infection in mice (68), those studies were performed in naïve animals, which lack pre-existing anti-WNV antibodies. As our passive transfer experiments suggested that FcγRIII contributed to E28-mediated protection, and NK cell ADCC requires FcγRIII (62), it seemed plausible that NK cells might be required for the survival benefit conferred by E28. However, mice depleted of NK cells showed no significant change in E28-mediated protection compared to those receiving an isotype control, non-depleting MAb. This was supported by evidence that E28 does not protect *C1q*$^{-/-}$ x *FcγRI*$^{-/-}$ mice from WNV infection (**Table S1**). These mice presumably have functional FcγRIII on their NK cells, and their NK cells were not sufficient for E28-mediated protection.

As complement and FcγRs also can promote phagocytosis of antibody-opsonized antigens, we speculated that a specific cell type capable of phagocytosis of viral particles

or infected cells conferred E28-mediated protection. After non-selectively depleting phagocytes from wild type mice with clodronate containing liposomes, E28 no longer reduced WNV viremia during the first two days of infection. Since clodronate-containing liposomes deplete several cell types with phagocytic potential (78), we tested the role of specific populations of phagocytes by antibody depletion. Gr-1 is expressed on neutrophils and inflammatory monocytes (21), both of which have phagocytic potential. As E28 MAb prophylaxis retained its ability to decrease viremia in mice treated with depleting concentrations of anti-Gr-1 antibody, neutrophils and inflammatory monocytes were likely not the key phagocytic cell types that conferred protection. While additional experiments are needed to absolutely identify the target cell type, our data collectively suggests that a complement and FcγR-expressing innate immune phagocyte (e.g., tissue macrophage or activated dendritic cell) mediates protection of E28 in mice.

While the detailed studies with E28 were informative from a mechanistic standpoint, it was important to confirm whether poorly neutralizing polyclonal antibodies shared the same phenotype. Purified polyclonal IgG from the serum of DENV-immune hamsters was E28-like, as it recognized SVP in a capture ELISA, was specific largely for the DII-FL epitope on WNV E protein, and was poorly neutralizing in both PRNT and RVP assays. Analogously, these cross-reactive polyclonal antibodies nonetheless protected wild type mice from WNV infection. The anti-WNV antibody repertoire in many human patients is skewed similarly toward the DII-FL epitope (54, 75), which is somewhat surprising given that MAbs recognizing this epitope neutralize WNV poorly and do not bind fully mature virus (51). In screening sera from humans with a remote history of DENV infection, a single prior exposure elicited a minor cross-reactive

antibody response, whereas individuals with evidence of multiple heterotypic infections perhaps unsurprisingly elicited higher titers of WNV reactive antibodies (M. Vogt and M. Diamond, unpublished results), which neutralized WNV poorly and bound the DII-FL epitope. The similarity of the DENV immune human sera to the protective hamster IgG suggests that immunodominant DII-FL antibodies elicited in response to natural DENV infection (16), while non-neutralizing against WNV and undetected by the PRNT assay, could still confer protection via Fc effector function mechanisms. While further epidemiologic studies are warranted, widespread exposure to DENV and other endemic flaviviruses in tropical America could result in production of polyclonal DII-FL-specific antibodies that fail to neutralize WNV infection by conventional PRNT analysis but still protect humans *in vivo*, and thus contribute to the lack of severe cases of human WNV infection in this region. Indeed, one recent serological surveillance study in Mexico supports this hypothesis (63).

Overall this study describes a mechanism for the protective effects *in vivo* of anti-WNV antibodies that are poorly neutralizing by *in vitro* assays. For WNV, both MAbs and polyclonal antibodies directed against the DII-FL epitope protected wild type mice from lethal WNV infection in vivo. This activity was dependent upon the Fc effector functions of the antibodies, and required phagocytic cells, C1q and FcγRIII. The ability of cross-reactive antibodies elicited by heterologous flavivirus infection to protect against WNV *in vivo* could explain the fewer than anticipated number of reported cases of WNV infection in Central and South America, and analogously, the historical observation of reduced numbers of SLEV or JEV infections in DENV-immune regions of the Americas and Southeast Asia (11, 31). Finally, the inability of the PRNT assay to predict the

functional capacity of specific classes of protective anti-flavivirus antibodies should give pause to its use as the definitive assay for measuring the immune response to the multitude of flavivirus vaccines that are in clinical testing or under development.

TABLE 1. MAb protection of 4 to 5 week-old wild type mice

MAb	Epitope on WNV E	Isotype	Neutralizing activity	MTD ± SD[a]	Survival (%)	*P* value
PBS	-	-	-	10.1 ± 2.1	8 of 57 (14%)	-
Control MAb (DENV2-E70, anti-DENV E)	-	IgG1	-	10.2 ± 2.1	4 of 20 (20%)	0.6
Control MAb (2H2 anti-DENV prM)	-	IgG2a	-	10.2 ± 1.6	3 of 30 (10%)	0.9
E24	DIII-LR	IgG2a	+++	15	28 of 29 (97%)	< 0.0001
E28	DII-FL	IgG1	-	11.1 ± 3.1	24 of 40 (60%)	< 0.0001
E34	DIII-LR	IgG1	+++	12	9 of 10 (90%)	< 0.0001
E53	DII-FL	IgG2a	+	10.4 ± 1.9	13 of 30 (43%)	0.003

Four to five week-old wild type mice were passively transferred 40 μg of the indicated MAbs one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Survival analysis was followed for 21 days and *P* values were determined using the log rank test as compared to PBS-treated mice. The isotype control MAbs react specifically with DENV but not WNV proteins (data not shown). Neutralization activity was scored according to data in **Fig 1A**: +++, strong neutralization; +, partial neutralization; and -, no neutralization. The epitope location on WNV E protein is based on prior published studies (53, 55).

[a] Mean time to death ± standard deviation of the mice that succumbed to infection.

TABLE 2. **MAb protection of 8 to 12 week-old mice lacking antibody effector functions**

A. *C1q*$^{-/-}$ mice

MAb	Dose (μg)	Epitope on WNV E	Isotype	MTD ± SD[a]	Survival (%)	*P* value
PBS	-	-	-	11.0 ± 0.6	5 of 12 (42%)	-
Control MAb (DENV2-E70, anti-DENV E)	40	-	IgG1	11.4 ± 1.7	6 of 16 (38%)	0.9
Control MAb (2H2 anti-DENV prM)	400	-	IgG2a	12.0 ± 2.3	3 of 8 (38%)	0.9
E24	40	DIII-LR	IgG2a	N.A.	12 of 12 (100%)	0.002
E24	400	DIII-LR	IgG2a	N.A.	11 of 11 (100%)	0.003
E28	40	DII-FL	IgG1	14.0 ± 2.0	7 of 10 (70%)	0.11
E34	40	DIII-LR	IgG1	17	8 of 9 (89%)	0.02
E53	40	DII-FL	IgG2a	13.0 ± 2.0	8 of 11 (73%)	0.09
E53	400	DII-FL	IgG2a	16	9 of 10 (90%)	0.02

B. *FcγR*$^{-/-}$

MAb	Dose (μg)	Epitope on WNV E	Isotype	MTD ± SD[a]	Survival (%)	*P* value
PBS	-	-	-	10.6 ± 2.0	3 of 15 (20%)	-
Control MAb	40	-	IgG1	11.4 ± 1.6	1 of 9 (11%)	0.9

(DENV2-E70, anti-DENV E)						
Control MAb (2H2 anti-DENV prM)	400	-	IgG2a	12.3 ± 3.6	5 of 12 (42%)	0.3
E24	40	DIII-LR	IgG2a	N.A.	10 of 10 (100%)	0.0001
E24	400	DIII-LR	IgG2a	N.A.	11 of 11 (100%)	< 0.0001
E28	40	DII-FL	IgG1	12.5 ± 1.0	7 of 11 (64%)	0.008
E34	40	DIII-LR	IgG1	N.A.	5 of 5 (100%)	0.006
E53	40	DII-FL	IgG2a	11.8 ± 1.6	4 of 10 (40%)	0.16
E53	400	DII-FL	IgG2a	9.8 ± 1.0	8 of 12 (67%)	0.04

C. *C1q*$^{-/-}$ x *FcγR*$^{-/-}$

MAb	**Dose (μg)**	**Epitope on WNV E**	**Isotype**	**MTD ± SD[a]**	**Survival (%)**	***P* value**
PBS	-	-	-	11.2 ± 1.1	1 of 25 (4%)	-
Control MAb (DENV2-E70, anti-DENV E)	40	-	IgG1	11.8 ± 1.6	2 of 17 (12%)	0.1
Control MAb (2H2 anti-DENV prM)	400	-	IgG2a	11.0 ± 2.0	1 of 10 (10%)	0.5
E24	40	DIII-LR	IgG2a	16.0 ± 2.6	14 of 17 (82%)	< 0.0001

E24	400	DIII-LR	IgG2a	N.A.	11 of 11 (100%)	< 0.0001
E28	40	DII-FL	IgG1	13.1 ± 2.2	1 of 15 (7%)	0.01
E28	400	DII-FL	IgG1	12.8 ± 1.8	0 of 8 (0%)	0.09
E34	40	DIII-LR	IgG1	15.0 ± 5.7	8 of 10 (80%)	< 0.0001
E53	40	DII-FL	IgG2a	12.9 ± 2.2	0 of 10 (0%)	0.08
E53	400	DII-FL	IgG2a	12.9 ± 3.1	0 of 8 (0%)	0.4

D. *C1q*$^{-/-}$ x *FcγRIII*$^{-/-}$

MAb	**Dose (μg)**	**Epitope on WNV E**	**Isotype**	**MTD ± SD[a]**	**Survival (%)**	***P* value**
PBS	-	-	-	11.8 ± 2.6	13 of 25 (52%)	-
Control MAb (DENV2-E70, anti-DENV E)	40	-	IgG1	12.0 ± 4.6	4 of 8 (50%)	> 0.9
E24	40	DIII-LR	IgG2a	N.A.	5 of 5 (100%)	0.07
E28	40	DII-FL	IgG1	11.3 ± 3.1	6 of 16 (38%)	0.3
E53	40	DII-FL	IgG2a	13.0 ± 3.3	16 of 24 (67%)	0.3

Eight to twelve week-old mice were passively transferred PBS or the indicated MAbs one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Survival was followed and *P* values were determined using the log rank test compared to PBS-treated mice. N.A. indicates not applicable as none of the animals succumbed to infection.

[a] Mean time to death ± standard deviation of the mice that succumbed to infection.

TABLE S1. **MAb protection of 8 to 12 week-old *C1q*$^{-/-}$ x *FcγRI*$^{-/-}$ mice**

MAb	Dose (μg)	Epitope on WNV E	Isotype	MTD ± SD[a]	Survival (%)	*P* value
PBS	-	-	-	11.4 ± 1.4	3 of 10 (30%)	-
Control MAb (DENV2-E70, anti-DENV E)	40	-	IgG1	12.3 ± 2.4	4 of 11 (36%)	0.6
E34	40	DIII-LR	IgG1	N.A.	5 of 5 (100%)	0.02
E28	40	DII-FL	IgG1	13.4 ± 1.8	7 of 17 (41%)	0.2
E53	40	DII-FL	IgG2a	11.9 ± 1.5	2 of 9 (22%)	> 0.9

Eight to twelve week-old mice were passively transferred PBS or the indicated MAbs one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Survival analysis was followed for 21 days and *P* values were determined using the log rank test compared to PBS-treated mice. N.A. indicates not applicable as none of the animals succumbed to infection.

[a] Mean time to death ± standard deviation of the mice that succumbed to infection.

FIGURE LEGENDS

Figure 1. **Neutralizing activity of MAbs in cell culture against different preparations of WNV. A, E, and F**. PRNT assay. E28 (DII-FL, IgG1), E53 (DII-FL, IgG2a), E24 (DIII-LR, IgG2a), and E34 (DIII-LR, IgG1) were tested for neutralization of (**A**) C6/36 cell, (**E**) Vero cell or (**F**) plasma-derived WNV (50 to 125 PFU) by classical PRNT assay on BHK21-15 cells. Data shown are combined results of two independent experiments in triplicate. The data is normalized to data from six control wells in each experiment with no MAb. **B.** RVP neutralization assay on Raji-DCSIGN-R cells. E28 was incubated with RVP prior to infection of Raji-DCSIGN-R cells. RVP were prepared normally (mixture of mature, immature, and partially mature) or in cells over-expressing the furin protease (mature) to create a more homogeneous population of mature virions. The data shown are the combined results of three independent experiments performed in duplicate, and the results are normalized to that from two control wells in each experiment with no MAb. **C**. WNV neutralization assay on Raji-DCSIGN-R cells. WNV (MOI of 0.001) derived from C6/36 cells, Vero cells, or plasma of infected *AG129* mice was mixed with medium (DMEM) or 30 μg/ml of MAbs E24 or E28 prior to infection of Raji-DCSIGN-R cells. One day later, cells were stained with Ch-E16 and processed by flow cytometry. The data shown are representative of three independent experiments performed in triplicate, and are normalized to that from three control wells for each virus with no MAb. **D**. A Western blot with anti-E (*top*) or anti-prM/M (*bottom*) antibodies using WNV derived from C6/36 cells, Vero cells, or plasma from infected *AG129* mice. For anti-E blot, an equivalent number of PFU were loaded as judged by plaque assay on BHK21-15 cells. **G.** Inhibition of WNV replication by MAbs in multi-step growth analysis. C6/36 cell-derived WNV was mixed with medium (DMEM) or 30 μg/ml of

MAbs E24 or E28 prior to infection of BHK21-15 cells. At the indicated time points, supernatant was harvested for titration by plaque assay. Data shown are combined data from three independent experiments performed in triplicate.

Figure 2. Protective effect of E28 MAb in mice. **A**. WNV burden in the serum of 5 week-old wild type C57BL/6 mice that were administered PBS or E28 (40 μg) via an intraperitoneal route one day prior to WNV infection (C6/36 cell-derived, 10^2 PFU) via a subcutaneous route. At the indicated days, serum was harvested and viral burden was determined by qRT-PCR assay. The data is expressed as genome equivalents per ml of serum and reflects 8 to 20 animals per condition per time point. Asterisks indicate values that are statistically significant (**, $P < 0.01$; ***, $P < 0.001$). **B**. WNV burden in the brain of 5 week-old wild type mice. Mice were treated as described above. At days 5 or 7 after WNV infection, brains were harvested and viral burdens were determined by plaque assay on BHK21-15 cells. The data is expressed as PFU per gram and reflects 8 to 10 animals per condition per time point. The following percentage of mice had viral burdens below detection (< 65 PFU/g): day 5, PBS - 0%; E28 – 40%; day 7, PBS – 0%; E28 – 25%. Asterisks indicate values that are statistically significant (*, $P < 0.05$). All analysis utilized the Mann-Whitney non-parametric t test.

Figure 3. Efficacy of different anti-WNV MAbs in *C1q*$^{-/-}$ and *Fc*γ*R*$^{-/-}$ mice. Eight to twelve week-old (**A**) *C1q*$^{-/-}$, (**B**) *Fc*γ*R*$^{-/-}$, or (**C**) *C1q*$^{-/-}$ x *Fc*γ*R*$^{-/-}$ C57BL/6 mice were passively transferred 40 μg of E24 (DIII-LR, IgG2a), E34 (DIII-LR, IgG1), E53 (DII-FL, IgG2a), E28 (DII-FL, IgG1), an isotype control (IgG1), or PBS one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Animals were followed for survival over a period of three weeks. The number of animals for each antibody condition ranged as

follows: *C1q*$^{-/-}$: 9 to 16; *Fc*γ*R*$^{-/-}$: 5 to 15; and *C1q*$^{-/-}$ x *Fc*γ*R*$^{-/-}$: 8 to 25. Statistically significant differences are described in the text and were compared to the PBS treated mice.

Figure 4. Functional activity of wild type or aglycosyl chimeric E28 MAbs in vitro and in vivo. **A**. A capture ELISA was used to detect binding of wild type or N297Q Ch-E28 MAbs to WNV SVP. Microtiter plates were coated with murine E24, incubated with SVP, and detected with increasing concentrations of the indicated chimeric (Ch-E28, N297Q Ch-E28, or Ch-E16) or control human IgG1 antibodies. One representative of three independent experiments is shown. **B**. PRNT assay. Ch-E28 and N297Q Ch-E28 were tested for neutralization of C6/36 cell-derived WNV by standard PRNT assay on BHK21-15 cells. Data shown are combined results of two independent experiments in triplicate. The data is normalized to data from six control wells in each experiment with no MAb. **C**. Four week-old wild type C57BL/6 mice were passively transferred PBS or 40 μg of Ch-E28 or N297Q Ch-E28E24 one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Animals were followed for survival over a period of three weeks. The number of animals for each antibody condition ranged from 13 to 19. Asterisks indicate values that are statistically significant by the log rank test (*, $P < 0.05$). **D**. WNV burden in the brain of 4 week-old wild type mice. Mice were treated as described above. At day 7 after WNV infection, brains were harvested and viral burdens were determined by plaque assay on BHK21-15 cells. The data is expressed as PFU per gram and reflects 22 animals per condition. The following percentage of mice had viral burdens below detection (< 100 PFU/g): PBS - 14%; Ch-E28 – 59%; N297Q Ch-E28 – 36%. Asterisks indicate values that are statistically significant by a one-way ANOVA (**, $P < 0.01$). The

viral burden between Ch-E28 and N297Q Ch-E28 approached statistical significance ($P = 0.05$).

Figure 5. Protective effect of E28 MAb in *RAG1*$^{-/-}$ mice. WNV burden in the serum of eight to twelve week-old congenic *RAG1*$^{-/-}$ C57BL/6 mice that were administered E28 (500 μg) or an isotype control MAb via an intraperitoneal route one day prior to WNV infection (C6/36 cell-derived, 1 PFU) via a subcutaneous route. At the indicated days, serum was harvested and viral burden was determined by qRT-PCR assay. The data is expressed as genome equivalents per ml of serum and reflects 5 to 10 animals per condition per time point. Asterisks indicate values that are statistically significant by the Mann-Whitney non-parametric t test (**, $P < 0.01$, ***, $P < 0.001$).

Figure 6. Effect of depletion of NK cells on protective activity of E28 MAb. NK cells were depleted from wild type mice after treatment with anti-NK1.1 antibody (100 μg) 2 days before and after infection with WNV. **A**. Depletion of NK cells was confirmed by flow cytometry after staining with anti-NKp46 and anti-CD3. **B**. WNV infection of NK cell-depleted mice. Sixteen wild type mice (five week-old) were treated with either anti-NK1.1 or an isotype control antibody (2H2, anti-DENV prM), administered E28 (40 μg), infected with WNV (C6/36 cell-derived, 10^2 PFU) and monitored for survival. No statistically significant differences in mortality were observed by the log rank test ($P > 0.6$).

Figure 7. Effect of depletion of phagocytes and neutrophils on protective activity of E28 MAb. Eight to twelve week-old wild type mice were administered E28 (500 μg) or an isotype control MAb via an intraperitoneal route one day prior to WNV infection (C6/36 cell-derived, 1 PFU) via a subcutaneous route. **A-B**. Depletion of phagocytes with

clodronate-containing liposomes. **A**. (*Left*) Gating strategy of $CD11b^{+}$ and $CD45^{+}$ cells by flow cytometry. (*Right*) Quantitative depletion of $CD11b^{+}$ and $CD45^{+}$ phagocyte cell populations in the spleen of $RAG1^{-/-}$ mice by clodronate liposomes. Depletion with clodronate-containing liposomes reduced the number of $CD45^{int}CD11b^{hi}$ and $CD45^{hi}CD11b^{int}$ by 74% and 91%, respectively. **B** Effect of clodronate or PBS liposomes on E28-mediated reduction in viremia. Wild type mice were treated with clodronate- or PBS-containing liposomes one day prior to and after infection with WNV. At the indicated days, serum was harvested and viral burden was determined by qRT-PCR assay. The data is expressed as genome equivalents per ml of serum and reflects 8 animals per condition per time point. Asterisks indicate values that are statistically significant (**, $P < 0.01$). **C-D**. Effect of depletion of neutrophils and inflammatory monocytes on protective activity of E28 MAb. Neutrophils and inflammatory monocytes were depleted from wild type mice after treatment with anti-Gr-1 antibody (250 μg) one day before infection with WNV. **C**. Depletion of neutrophils and inflammatory monocytes was confirmed by flow cytometry after staining with anti-Ly6C and Ly6G. **D**. Effect of neutrophil and inflammatory monocyte depletion on E28-mediated reduction in viremia. At day 2 after infection, serum was harvested and viral burden was determined by qRT-PCR assay. The data is expressed as genome equivalents per ml of serum and reflects 8 animals per condition per time point. Asterisks indicate values that are statistically significant (*, $P < 0.05$; ***, $P < 0.001$). All analyses utilized the Mann-Whitney test.

Figure 8. Functional activity in vitro and in vivo of polyclonal IgG derived from naïve or DENV-2 infected hamsters. **A**. A capture ELISA was used to detect binding of

naive or DENV-2 immune polyclonal IgG to WNV SVP. Microtiter plates were coated with murine E24, incubated with SVP, and detected with increasing concentrations of the indicated affinity-purified polyclonal hamster IgG. One representative of three independent experiments is shown. **B**. PRNT assay. Naive and DENV-2 immune polyclonal IgG were tested for neutralization of C6/36 cell-derived WNV by PRNT assay on BHK21-15 cells. Data shown are combined results of two independent experiments performed in triplicate. The data is normalized to data from six control wells in each experiment with no MAb. **C**. RVP neutralization assay on Raji-DCSIGN-R cells. Increasing concentrations of naive or DENV-2 immune polyclonal IgG were incubated with RVP for one hour prior to infection of Raji-DCSIGN-R cells. RVP were prepared normally (mixture of mature, immature, and partially mature) or in cells over-expressing the furin protease (mature) to create virions of different maturation states. Data shown are representative results of two independent experiments performed in duplicate. **D**. Epitope specifity of DENV-2 immune hamster IgG against WNV E protein. Comparison of antibody titer from DENV-2 immune hamster for wild type (E-WT) and DII-FL loss-of-function variants (E-W101R and E-quadruple (Quad) mutant). Titers of antibodies were compared from individual DENV-2 infected hamsters. Note, the plating density of E-WT, E-W101R, and E-quadruple mutant was equivalent as no difference in binding was observed with E24 (DIII-LR) MAb when tested in parallel (data not shown). Asterisks indicate values that are statistically significant by the student's paired t test (**, $P < 0.01$). **E**. Four to five week-old wild type C57BL/6 mice were passively transferred PBS or 400 μg of pooled naïve or DENV-2 immune affinity-purified IgG one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Animals were followed for survival

over a period of three weeks. The number of animals for each condition ranged from 10 to 15. Asterisks indicate values that are statistically significant by the log rank test (*, $P < 0.05$).

Figure 9. Sera from humans with a history of multiple DENV infections contain cross-reactive, poorly neutralizing antibodies directed against the DII-FL epitope. The cross-reactivity of these human immune sera for DENV is described in the Materials and Methods. **A**. Epitope specificity of DENV immune human serum against WNV E protein. Comparison of antibody titer from DENV immune human serum for wild type (E-WT) and DII-FL loss-of-function variants (E-W101R and E-quadruple (Quad) mutant). Note the plating density of E-WT, E-W101R, and E-quadruple mutant was equivalent as no difference in binding was observed with Ch-E16 (DIII-LR) MAb when tested in parallel (data not shown). Asterisks indicate values that are statistically significant by the student's paired t test (*, $P < 0.05$; **, $P < 0.01$). **B**. WNV PRNT assay. DENV immune human sera from six individuals with a history of heterotypic infections was tested for neutralization of C6/36 cell-derived WNV by PRNT assay on BHK21-15 cells. Data shown are combined results of two independent experiments performed in triplicate. The data is normalized to data from 12 control wells in each experiment with naïve human serum.

FIGURE 1

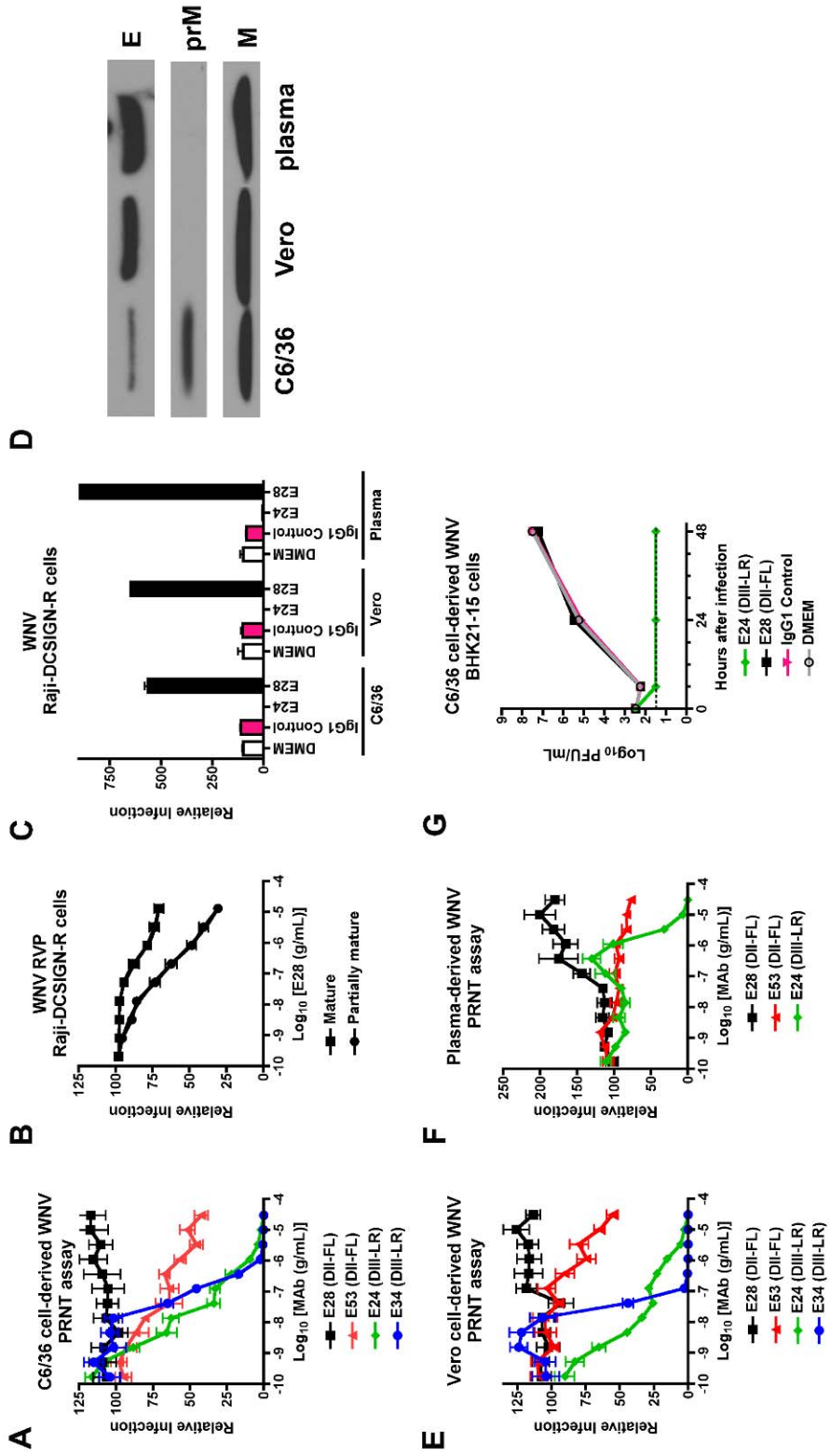

A
C6/36 cell-derived WNV PRNT assay
Relative Infection
Log10 [MAb (g/mL)]
E28 (DII-FL)
E53 (DII-FL)
E24 (DIII-LR)
E34 (DIII-LR)
B
WNV RVP Raji-DCSIGN-R cells
Relative Infection
Log10 [E28 (g/mL)]
Mature
Partially mature
C
WNV Raji-DCSIGN-R cells
Relative Infection
DMEM
IgG1 Control
E24
E28
C6/36
Vero
Plasma
D
E
prM
M
C6/36
Vero
plasma
E
Vero cell-derived WNV PRNT assay
Relative Infection
Log10 [MAb (g/mL)]
E28 (DII-FL)
E53 (DII-FL)
E24 (DIII-LR)
E34 (DIII-LR)
F
Plasma-derived WNV PRNT assay
Relative Infection
Log10 [MAb (g/mL)]
E28 (DII-FL)
E53 (DII-FL)
E24 (DIII-LR)
G
C6/36 cell-derived WNV BHK21-15 cells
Log10 PFU/mL
Hours after infection
E24 (DIII-LR)
E28 (DII-FL)
IgG1 Control
DMEM

FIGURE 2

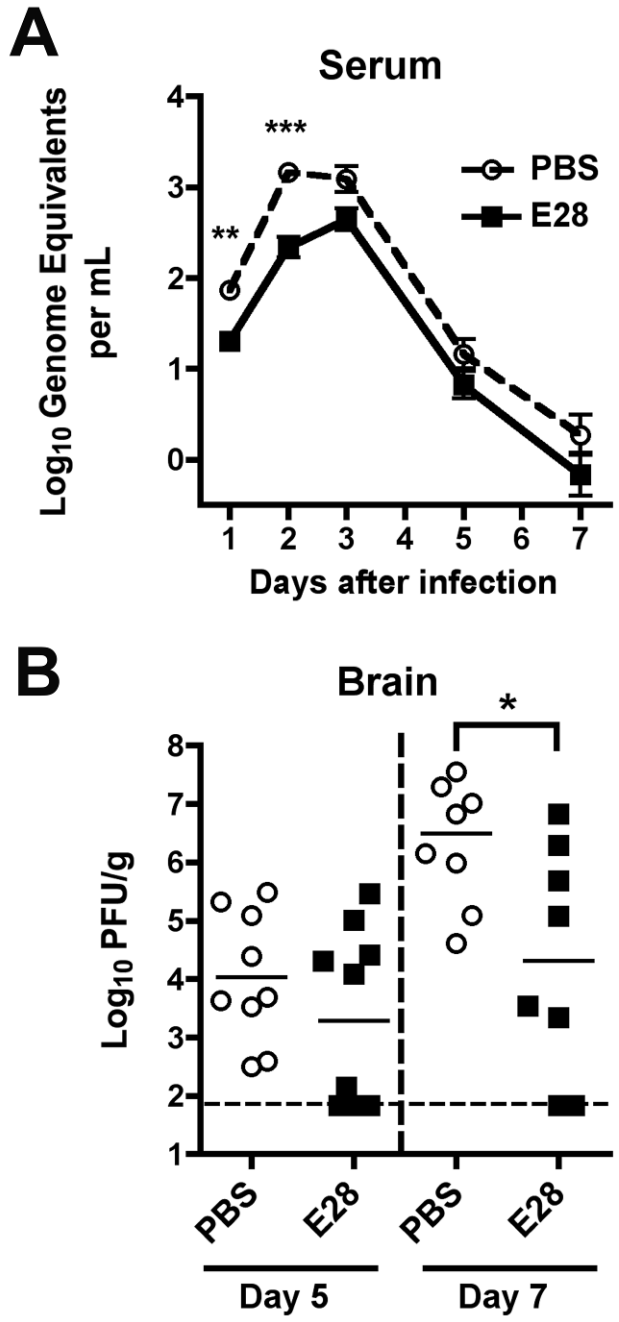

A
Serum
Log10 Genome Equivalents per mL
PBS
E28
**
***
Days after infection
B
Brain
Log10 PFU/g
*
PBS
E28
PBS
E28
Day 5
Day 7

FIGURE 3

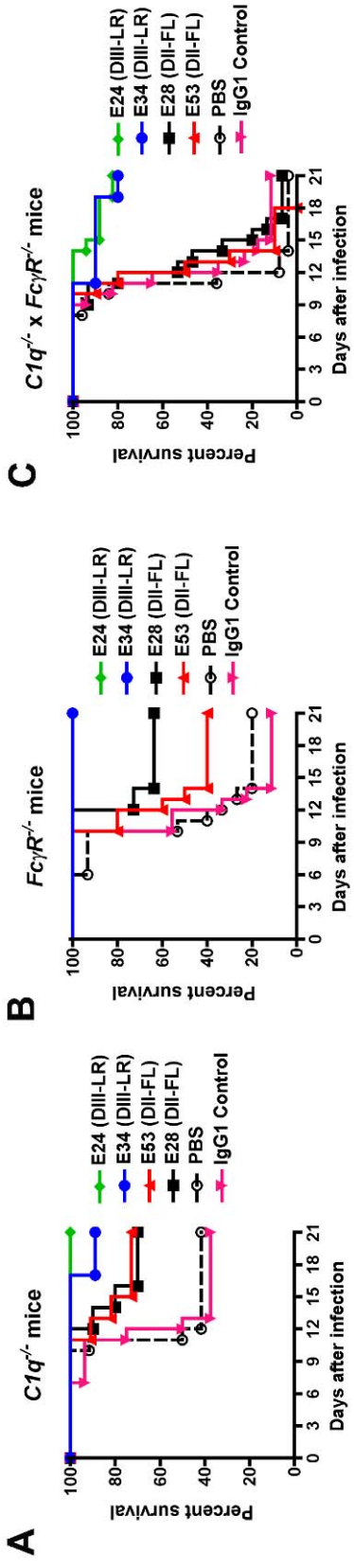

A
C1q-/- mice
B
FcγR-/- mice
C
C1q-/- x FcγR-/- mice
Percent survival
Days after infection
E24 (DIII-LR)
E34 (DIII-LR)
E53 (DII-FL)
E28 (DII-FL)
PBS
IgG1 Control

FIGURE 4

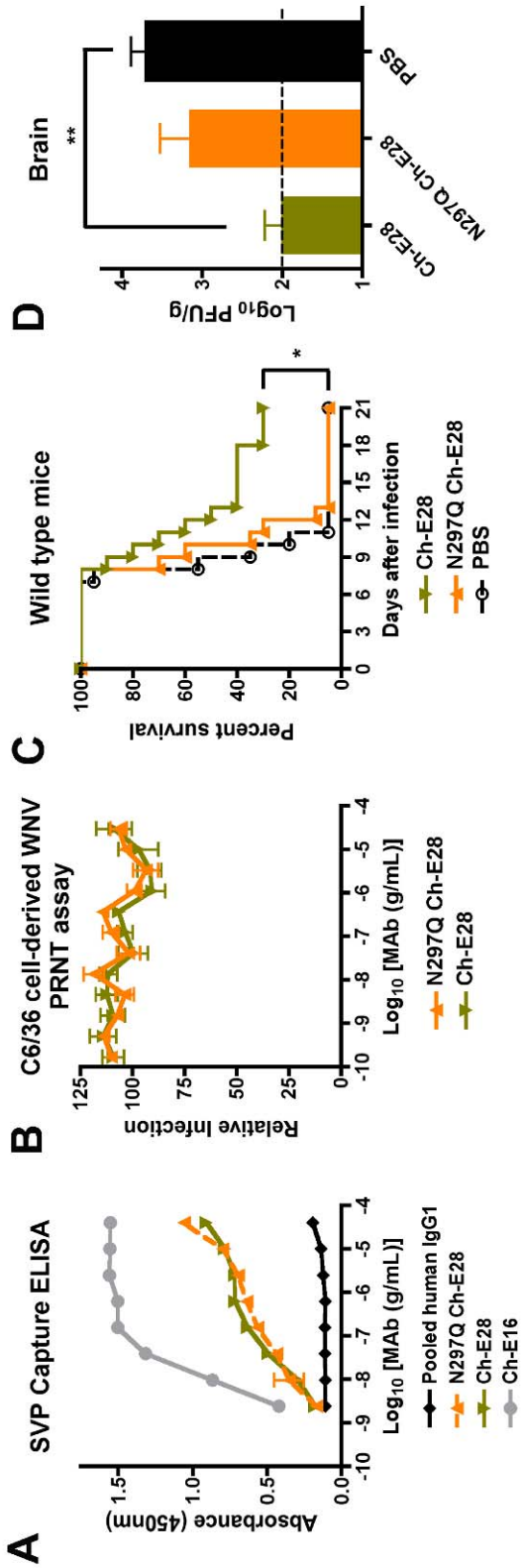

A
SVP Capture ELISA
Absorbance (450nm)
Log10 [MAb (g/mL)]
Pooled human IgG1
N297Q Ch-E28
Ch-E28
Ch-E16
B
C6/36 cell-derived WNV PRNT assay
Relative Infection
Log10 [MAb (g/mL)]
N297Q Ch-E28
Ch-E28
C
Wild type mice
Percent survival
Days after infection
Ch-E28
N297Q Ch-E28
PBS
*
D
Brain
**
Log10 PFU/g
Ch-E28
N297Q Ch-E28
PBS

FIGURE 5

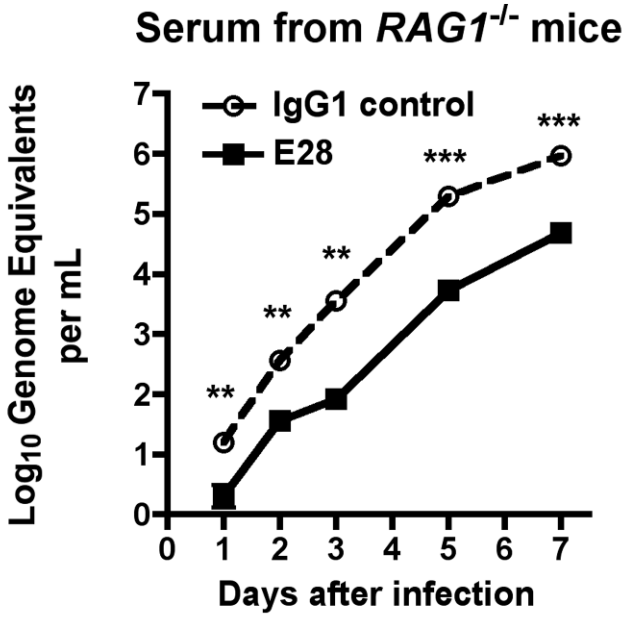

Serum from RAG1-/- mice
IgG1 control
E28
Log10 Genome Equivalents per mL
Days after infection
0
1
2
3
4
5
6
7
**
**
**
***
***

FIGURE 6

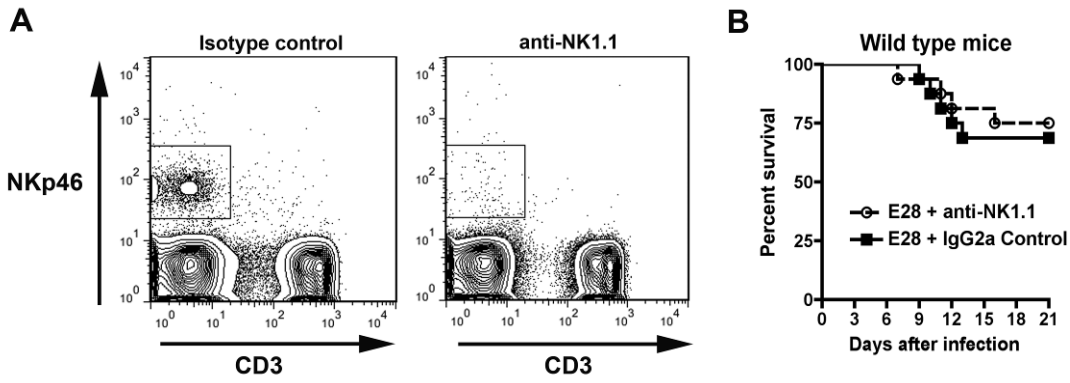

A
Isotype control
anti-NK1.1
NKp46
CD3
CD3
B
Wild type mice
Percent survival
E28 + anti-NK1.1
E28 + IgG2a Control
Days after infection

FIGURE 7

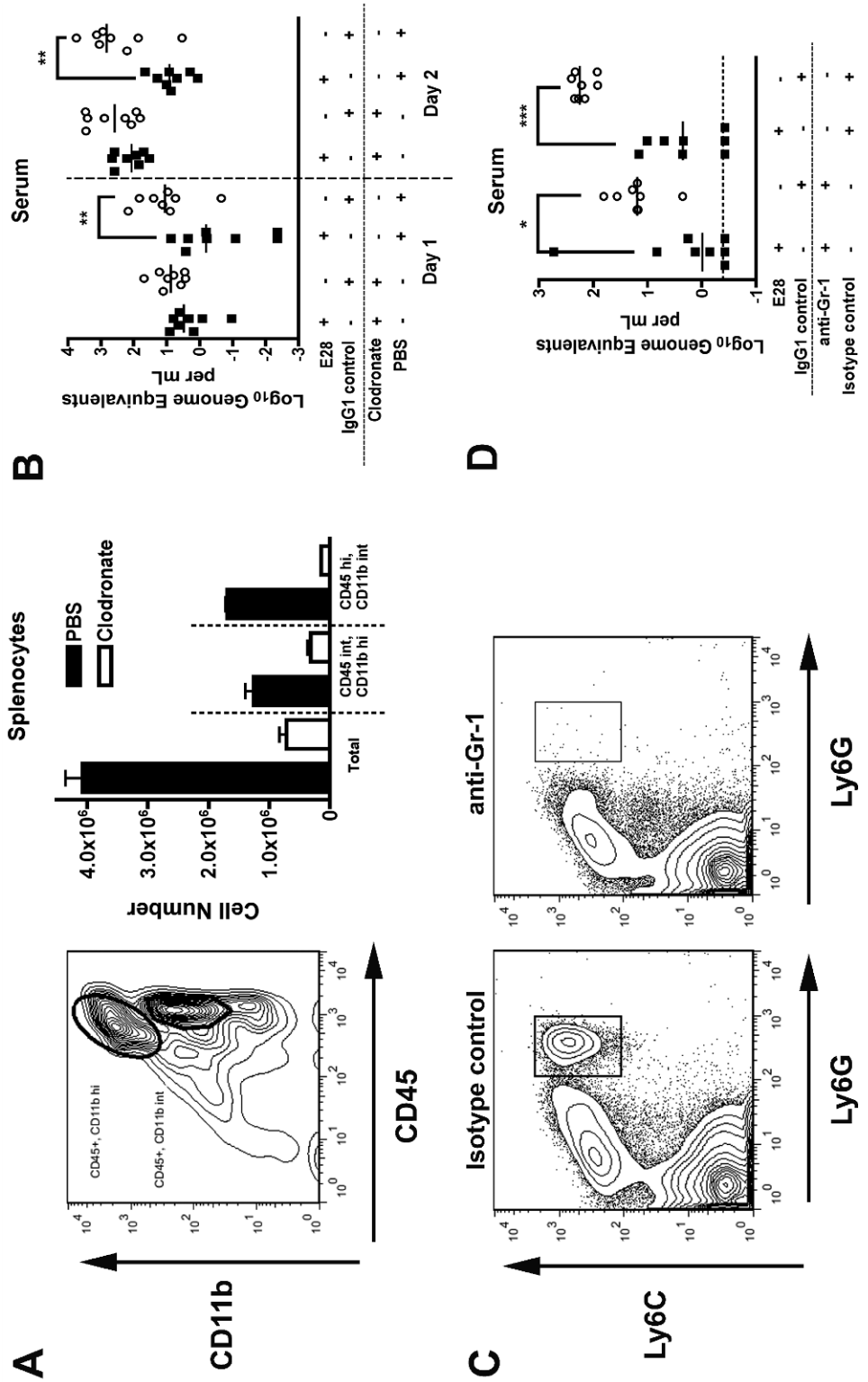

A
Splenocytes
PBS
Clodronate
Cell Number
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
Total
CD45 int, CD11b hi
CD45 hi, CD11b int
CD11b
CD45
CD45+, CD11b hi
CD45+, CD11b int
B
Serum
Log10 Genome Equivalents per mL
**
**
E28
IgG1 control
Clodronate
PBS
Day 1
Day 2
C
Isotype control
anti-Gr-1
Ly6C
Ly6G
Ly6G
D
Serum
Log10 Genome Equivalents per mL
*
***
E28
IgG1 control
anti-Gr-1
Isotype control

FIGURE 8

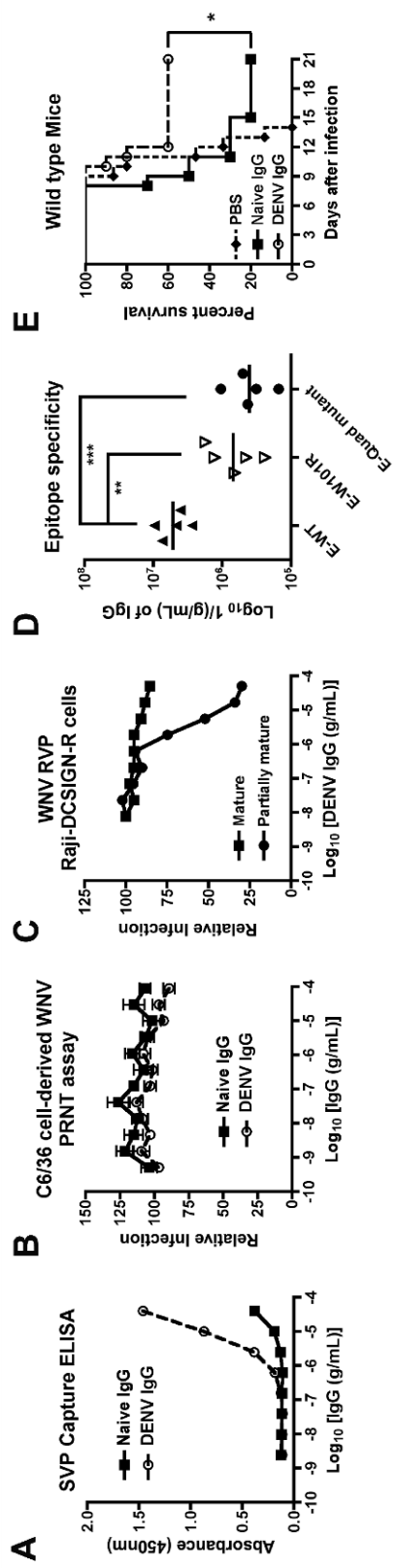
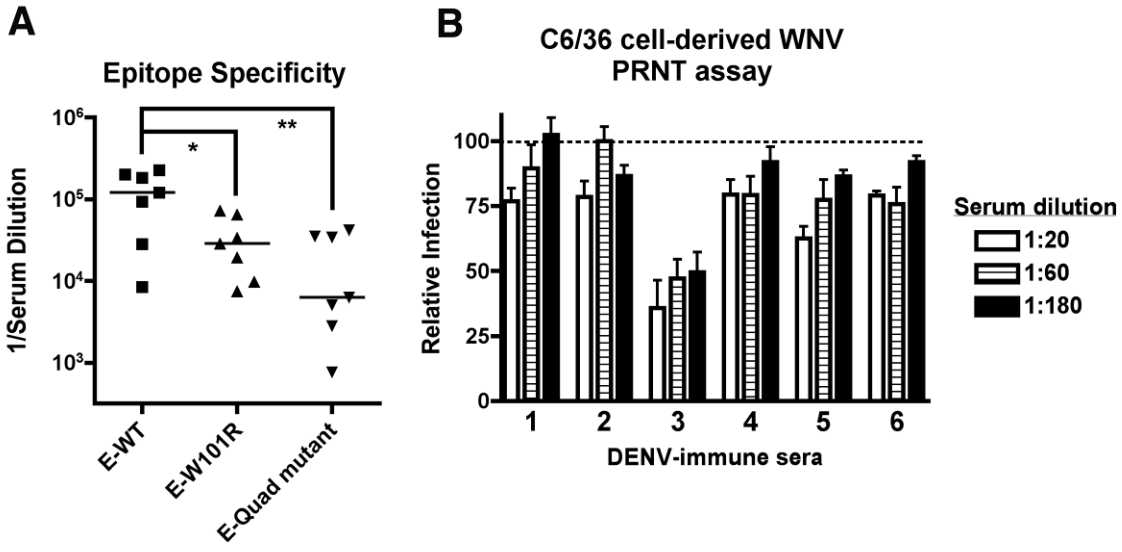

A
SVP Capture ELISA
Naive IgG
DENV IgG
Absorbance (450nm)
Log10 [IgG (g/mL)]
B
C6/36 cell-derived WNV PRNT assay
Naive IgG
DENV IgG
Relative Infection
Log10 [IgG (g/mL)]
C
WNV RVP Raji-DCSIGN-R cells
Mature
Partially mature
Relative Infection
Log10 [DENV IgG (g/mL)]
D
Epitope specificity
Log10 1/(g/mL) of IgG
E-WT
E-W101R
E-Quad mutant
E
Wild type Mice
PBS
Naive IgG
DENV IgG
Percent survival
Days after infection

FIGURE 9


A
Epitope Specificity
1/Serum Dilution
10^6
10^5
10^4
10^3
*
**
E-WT
E-W101R
E-Quad mutant
B
C6/36 cell-derived WNV
PRNT assay
Relative Infection
100
75
50
25
0
1
2
3
4
5
6
DENV-immune sera
Serum dilution
1:20
1:60
1:180

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Chapter IV

Conclusions and Future Directions

Conclusions

Prior to this thesis, extensive work had characterized the neutralizing antibody response to WNV. E protein reactive antibodies were determined immunodominant in the neutralizing response (4, 39), and the characteristics of antibodies binding to many specific epitopes on the E protein were defined (1, 6, 11, 27, 29, 33). MAbs binding to the DIII-LR epitope were consistently the most strongly neutralizing antibodies. These antibodies neutralize mature and immature virions equally (25), require only a small fraction of their available epitopes to be bound within a specific virion to eliminate infectivity (15, 26, 31), and function by steric inhibition of the pH-dependent E protein rearrangement necessary for fusion of the virion with the late endosomal membrane (26, 36). The neutralizing antibody repertoire of WNV-infected mice was dominantly directed toward the DIII-LR epitope, however studies with sera from convalescent WNV infected humans revealed that few neutralizing antibodies were directed toward this epitope (28). The absence of the most strongly neutralizing antibodies was surprising, as the antibody response is necessary for the resolution of WNV infection (7, 8), ~80% of human infections are asymptomatic (22), and the antibody response is protective against secondary challenge (7). A further unexpected result of these human studies was that the DII-FL epitope was immunodominant in WNV convalescent sera (28), as MAbs directed to this epitope fail to recognize fully mature WNV virions efficiently (25) and accordingly neutralize infection poorly in vitro (29). The work in this thesis was aimed at determining the significance of this apparent discrepancy between the ability of a polyclonal antibody repertoire consisting primarily of poorly neutralizing antibodies to efficiently limit the pathogenesis of WNV infection in vivo.

The first approach undertaken in this thesis challenged the assumption that the human antibody repertoire was poorly neutralizing. We hypothesized that strongly neutralizing antibodies were indeed generated, but were directed to cryptic epitopes on the E protein or non-E epitopes that went undetected based on limitations of the epitope based diagnostics used. This seemed likely because the WNV virion is composed of 90 E dimers, and each E protein exists in one of three different symmetry environments (23), none of which are likely present in recombinant protein ELISA assays used to quantify epitope specificity (28). This led us to characterize in detail two WNV-specific human MAbs, CR4348 and CR4354. These MAbs were isolated from B cell populations of convalescent patients by screening for binding to different forms of WNV antigen (37). Both MAbs strongly neutralized WNV infection of cultured cells and protected mice against lethal infection in vivo, yet they poorly recognized recombinant forms of E protein. Instead, we found that CR4348 and CR4354 bound determinants on intact WNV virions and subviral particles in a pH-sensitive manner, and neutralization was altered by mutations at the dimer interface in domain II and the hinge between domains I and II, respectively. CR4348 and CR4354 human MAbs neutralized infection at a post-attachment step in the viral lifecycle, likely by inhibiting acid-induced fusion within the endosome. Further studies with CR4354 determined that neutralization was not dependent upon bivalency of the MAb. Collaborators utilized cryo-electron microscopy (EM) of virions saturated with CR4354 Fab fragments to confirm binding to the DI-DII hinge epitope. These studies identified a novel mechanism of neutralization: tethering together E proteins from different symmetry environments to prevent the dissociation of E dimers necessary for fusion (16).

Based on historical examples of the ability of antiviral antibodies that neutralize poorly in vitro to protect against infection in animals (2, 10, 14, 18, 19, 21, 24, 29, 34, 38), the second approach of this thesis was to study the functional properties in vivo of the immunodominant but poorly neutralizing DII-FL antibodies. We showed that while passive transfer of poorly neutralizing MAbs and polyclonal antibodies against the DII-FL epitope protect against lethal WNV infection in wild type mice, they fail to protect mice lacking activating FcγRs and the complement opsonin C1q. Consistent with this, an aglycosyl chimeric mouse-human DII-FL MAb (E28) variant that lacked the ability to engage FcγR and C1q also did not protect against WNV infection in wild type mice. Using a series of immunodeficient mice and antibody depletions of individual immune cell populations, we demonstrated that the non-neutralizing DII-FL MAb E28 did not require T, B, or NK cells, inflammatory monocytes, or neutrophils for in vivo protection. Rather, E28 MAb treatment decreased viral load in the serum early in the course of infection, which resulted in blunted dissemination to the brain, an effect that required phagocytic cells, C1q and FcγRIII (CD16). Finally, we demonstrated that human and hamster polyclonal antibody responses after DENV infection that cross-react with WNV are generally poorly neutralizing and skewed to the DII-FL epitope, and that passive transfer of IgG purified from DENV-immune hamsters also protected mice from lethal WNV infection.

The work in this thesis highlights the neutralization potential of antibodies not accounted for by current diagnostic assays and enhances our understanding of the functional significance of immunodominant poorly-neutralizing antibodies in the polyclonal human anti-flavivirus response. These studies underscore the limitations of

many common applications of in vitro assays: the initial identification of antiviral MAbs by their ability to bind to non-native viral antigens and the usage of surrogate markers of protection such as cell-based neutralization assays as the definitive assay for measuring the immune response to vaccines. Finally, this work could explain the low number of infections by flaviviruses of the JEV serocomplex (WNV, JEV, and SLEV) in regions of the world with a high prevalence of DENV immunity (3, 12, 17, 30) due to Fc-mediated protection by cross-reactive poorly-neutralizing DII-FL antibodies, which may escape detection in conventional neutralization tests.

Future Directions

The studies with human MAbs CR4348 and CR4354 presented strong evidence for their epitopes localizing to the DII dimer interface and DI-DII hinge interface, respectively, but lacked definitive proof. Two structural approaches to determine antibody epitopes are co-crystallization with antigen for x-ray crystallography and binding to virions at saturating concentrations for cryo-EM, both utilizing Fab fragments to eliminate confounding structural mobility. Our collaborators performed cryo-EM studies with CR4354 Fab fragments, confirming that it binds to the DI-DII hinge epitope (16). Each asymmetric unit on the pseudo-icosahedral surface of the WNV virion contains three E proteins, one in each symmetry environment (2-, 3-, and 5-fold axes of symmetry) (23). The CR4354 Fab bound at two E proteins within the asymmetric unit (120 total sites per virion). Interetsingly,, the Fab electron density was observed over the DI-DII hinge of E in one environment as well as over sites in DIII of E in a second environment (16). This suggested that CR4354 may function to lock neighboring E

proteins together, thereby preventing the dissociation of E protein dimers at low pH in the late endosome to prevent fusion and infection. Unexpected results also were obtained in a separate study using similar methods to observe the binding of neutralizing 1A1D-2 Fab to DENV-2 virions, which trapped the virion in a different structural configuration (20). While we hypothesize that CR4348 binds across E protein dimers, definitive cryo-EM studies are required to establish a structural mechanism of inhibition.

Another alternative for epitope confirmation is to co-crystallize recombinant E protein with Fab fragments of CR4348 and CR4354. One potential complication to these studies is the monomeric nature of WNV E, both in solution and in crystals (13, 26), and neither of these human MAb appreciably bound to purified protein. Nonetheless, it remains possible that the addition of Fab to WNV E under crystallization conditions could drive dimer formation, especially with CR4348 Fab, which likely binds across the E dimer on the virion surface.

Even if strongly neutralizing MAbs that bind to CR4348 or CR4354-like epitopes are a significant part of the human repertoire, antibodies binding to these epitopes may go undetected using existing methods for defining the repertoire of antibodies in WNV convalescent humans (28). This indicates a need for new approaches to define the antibody repertoire of WNV immune humans with assays capable of detecting these epitopes. Although the methodology of measuring binding titers by ELISA to wild type antigen and quantitating the loss of binding to mutant antigen can still be utilized, in parallel, experiments with wild type or variant intact virions or subviral particles appear to be required.

Any new studies of the human antibody repertoire should also include a comparison of people who experienced asymptomatic versus symptomatic WNV infection. Since ~80% of humans do not experience any signs or symptoms of infection (22), studies with convalescent sera may not be representative of the typical human antibody response. This could explain the observed predominance of poorly neutralizing DII-FL antibodies, as these measurements could have focused on an ineffective repertoire of antibodies. People with asymptomatic infections are frequently identified in screenings of blood donors in WNV endemic areas, and cohorts of these individuals have been identified. Studies of the anti-E antibody repertoire of their sera may reveal that the majority of humans actually generate a more effective antibody response against strongly neutralizing epitopes.

The studies in this thesis showing an Fc dependent mechanism of protection against WNV infection by poorly neutralizing DII-FL MAb E28 provide a deeper understanding of the importance of these antibodies within the polyclonal immune repertoire. However, further studies are needed to define these Fc effector mechanisms more specifically. We have identified phagocytes as the cell type that contributes most to this effect. While many cells have phagocytic capacity, macrophages seem likely to mediate FcγR-dependent protection in vivo based on the following lines of evidence: macrophages do not not express Gr-1; FcγRI, FcγRIII and likely complement receptors, which are all expressed by macrophages, are necessary for protection; and clodronate liposomes treatment depleted $CD45^{+}CD11b^{+}$ cells. The classic macrophage deficient *op/op* mouse strain, which does not produce macrophage colony stimulating factor, is technically challenging because these osteopetrotic mice have myriad health concerns

(40), which would complicate the interpretation of their response to WNV infection. However, a new strain of *op/op* mice with restored osteoclast function could be a useful model to investigate the necessity of macrophages for E28 MAb mediated protection from WNV.

Antibodies that bind the DII-FL epitope are highly cross-reactive among flaviviruses (5, 6, 9, 29). The ability of DII-FL antibodies to protect against WNV led us to hypothesize a role for immunity generated against DENV infection in protecting humans from infection by viruses in the JEV serocomplex. Historically, areas of high DENV prevalence in Southeast Asia and the Americas have experienced low levels of infection by SLEV and JEV (3, 12). This could explain why WNV spread so rapidly through the United States and into Canada, yet has failed to expand cause human disease in Mexico, Central and South America despite the migration of birds that harbor WNV into these areas and appropriate mosquito vectors for transmission (17, 30). Further epidemiologic studies are warranted to define whether the inverse relationship between DENV and WNV prevalence in Latin America is dependent upon pre-existing DII-FL antibodies elicited by DENV infection. One such recent, large scale study in northern Mexico supports our hypothesis, although it did not test the epitope specificity of the potentially protective cross-reactive antibodies (32). Further, if DENV elicited DII-FL antibodies can indeed protect from infection by JEV serocomplex viruses, these antibodies may affect the immunogenicity of any attenuated vaccine strains. Existing JEV and even more distantly related YFV vaccine strains could be used to challenge DENV immune and naive individuals to observe for effects of cross-reactive antibodies. Finally, the ability of hamsters to elicit predominantly DII-FL antibodies in response to flavivirus

infection indicates they are a possible alternative model to mice for the study of flavivirus infection, especially for studies of the humoral immune response.

An interesting observation of the in vitro activities of E28 with WNV in cell culture is that this MAb was capable of both neutralization and enhancement of infection. E28 MAb poorly neutralized RVP, but in the PRNT assay with C6/36 and Vero cell-derived WNV it subtly enhanced infection; E28 markedly enhanced infection with plasma-derived WNV in the PRNT assay, which utilizes BHK21-15 cells, and with all WNV sources on Raji-DCSIGN-R cells. We have preliminary data that another anti-E MAb, E87, enhances C6/36 cell-derived WNV infection in the PRNT assay (**Fig 1A**) and in multi-step growth analysis (**Fig 1B**). Classical antibody-dependent enhancement (ADE) of infection in vitro is a reasonably well understood phenomenon, one involving the infection of FcγR bearing cells (35). However, the experiments with E28 and E87 used BHK21-15 cells, which lack expression of FcγRs. To further confirm FcγR-independence, $F(ab')_2$ fragments could be used in vitro. As with E28, E87 prophylaxis of mice also protected from WNV infection (**Fig 1C**), so any potential enhancing effects of these MAbs in vivo may be overcome by their protective Fc effector functions. Therefore, further study of FcγR-independent ADE is unlikely to provide insight on the natural course of WNV infection. Nonetheless, these studies are warranted as understanding the mechanism of FcγR-independent ADE in cell culture could increase our comprehension of the step(s) in the viral life cycle that are affected, especially if they were coupled with structural studies. One hypothesis that is intriguing is that some of these MAbs could trap WNV in a conformational state that is more infectious, either by enhancing attachment or entry or by stabilizing virus from degradation. Alternatively,

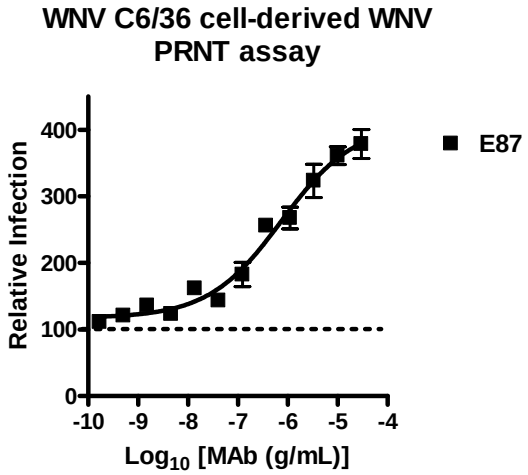
MAbs of this class could enhance infectivity by promoting formation of immune complexes and/or viral aggregates.

FIGURE LEGEND

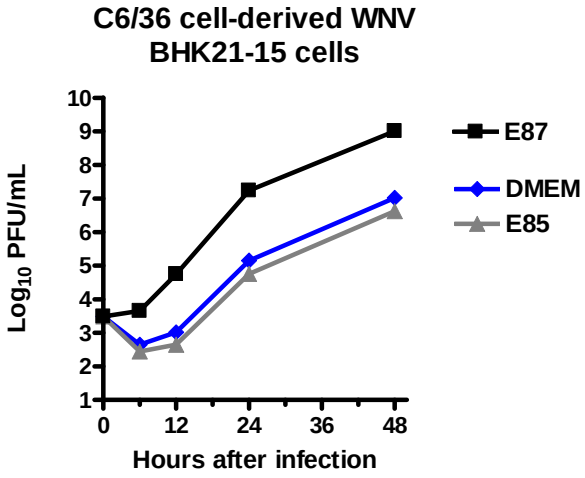
Figure 1. Effects of E87 MAb on WNV infection in vitro and in vivo. A. PRNT assay. Dilutions of E87 MAb were incubated with C6/36 cell-derived WNV for one hour at 37°C, then added to a monolayer of BHK21-15 cells for one hour at 37°C, then overlaid with agarose-containing medium and incubated at 37°C for 3 days before fixation with 10% formaldehyde and visualization of plaques by staining with crystal violet. Data shown are from a single experiment in triplicate. The data is normalized to data from three control wells with no MAb. **B.** Multi-step growth analysis. C6/36 cell-derived WNV was mixed 1:1 with medium (DMEM) or hybridoma supernatants of MAbs E85 or E87 for one hour at 37°C prior to infection of BHK21-15 cells at a MOI of 0.01. At the indicated time points, supernatant was harvested for titration by plaque assay. Data shown are from a single experiment. **C.** Effect of E87 prophylaxis in wild type mice. Five week old C57BL/6 mice were passively transferred 400 μg of E87 (n=10) or PBS (n=32) one day prior to infection with 10^2 PFU of C6/36 cell-derived WNV. Animals were followed for survival over a period of three weeks. Survival curves were significantly different as determined by the log rank test ($P = 0.0005$).

FIGURE 1

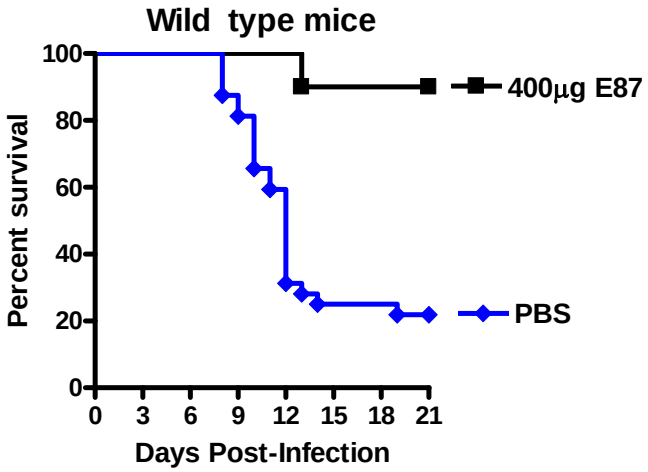
A.


WNV C6/36 cell-derived WNV
PRNT assay
Relative Infection
400
300
200
100
0
-10
-9
-8
-7
-6
-5
-4
Log10 [MAb (g/mL)]
E87

B.


C6/36 cell-derived WNV
BHK21-15 cells
Log10 PFU/mL
10
9
8
7
6
5
4
3
2
1
0
12
24
36
48
Hours after infection
E87
DMEM
E85

C.


Wild type mice
Percent survival
100
80
60
40
20
0
0
3
6
9
12
15
18
21
Days Post-Infection
400µg E87
PBS

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Appendix A

Development of Resistance to Passive Therapy with a Potently Neutralizing Humanized Monoclonal Antibody against West Nile Virus

This chapter is reprinted essentially as published in The Journal of Infectious Diseases.

Shuliu Zhang[*], Matthew R. Vogt[*], Theodore Oliphant, Michael Engle, Evgeniy I. Bovshik, Michael S. Diamond, and David W. C. Beasley. Journal of Infectious Diseases. 2009. 200 (2): 202-5.

*These authors contributed equally to the study.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus that has emerged as the primary cause of epidemic encephalitis in the United States. Although case numbers have declined somewhat since the peak activity of 2002 and 2003, virus transmission continues throughout the continental United States and thousands of cases of neurological disease have been reported each year since 2004. At present there is no WNV vaccine or antiviral therapy approved for use in humans, although promising treatment results were observed in case reports using purified immunoglobulin derived from Israeli donors [1, 2].

The potential of passive immunotherapy for treatment of WNV infection has led to the development and evaluation of potently neutralizing human or humanized mouse monoclonal antibodies (MAbs) [3, 4]. One therapeutic candidate, humanized E16 (hE16), binds to a highly conserved epitope on the upper lateral ridge of WNV envelope (E) protein domain III (E-DIII) and neutralizes WNV at low stoichiometric occupancy, apparently by inhibiting conformational changes in E that are required for fusion of the virus with host cell membranes [3, 5, 6]. However, mapping studies by several groups have identified mutations in the hE16 epitope that significantly reduce or abolish binding of this and other neutralizing WNV MAbs [3, 7-9]. A few of these mutations are found in WNV strains isolated in the field [8], suggesting that the efficacy of hE16 could be limited. Furthermore, flaviviruses have error-prone genome replication, resulting in significant genetic diversity within any individual isolate. Indeed, this property has been used as the basis for *in vitro* selection of variants resistant to a particular selective pressure, such as that imposed by antiviral inhibitors or neutralizing MAbs. The potential

for *in vivo* selection of MAb-resistant variants of flaviviruses has not yet been examined in detail.

In this study, we assessed (1) the potential for WNV strains encoding engineered mutations in the hE16 binding site to resist passive immunotherapy with hE16 in two mouse models of WNV neuroinvasive disease, and (2) the potential for neutralization-resistant variants to be selected *in vivo* during hE16 treatment.

MATERIALS AND METHODS

All WNV strains and infectious clone-derived variants used in this study (Table 1 and Results) were grown and plaque titrated on Vero cells. Neutralization assays were performed on Vero or BHK-21 cells, as described elsewhere [3]. RNA extractions, reverse-transcription polymerase chain reaction, and nucleotide sequencing of the pre-membrane (prM) and E coding regions of WNV genomes were performed using protocols and primers that have been described elsewhere [7].

Swiss Webster mice (female; 3-4 weeks of age) were obtained from Harlan Laboratories, and wild type and congenic *RAG* C57BL/6 mice (female; 5 weeks of age) were obtained from The Jackson Laboratory. All mice were housed in animal biosafety level 3 facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and experiments were conducted under protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch or Washington University School of Medicine. Details of individual passive-protection experiments are described below.

RESULTS

Previous crystallographic and epitope mapping studies have suggested that hE16 has key contacts at residues 307, 330, and 332 of the WNV E protein [3, 5]. The ability of hE16 to neutralize selected WNV strains and NY99 infectious clone-derived variants encoding single amino acid changes at these residues was initially assessed by a plaque reduction neutralization assay on Vero cells. These viruses have been shown to variably escape neutralizing MAbs [7, 8]. Notably, only the mutation T332K resulted in a substantial loss of hE16 neutralizing activity, whereas other mutations (K307R, T330I, T332A/M) showed only modest changes in neutralization compared to the wild-type lineage 1 NY99 virus (**Fig 1A**). Lineage 2 South African strain H442 (SA58), isolated in 1958 from a human patient, normally encodes a lysine at residue 332 and has been reported to be resistant to neutralization by several E-DIII-reactive antibodies raised against NY99 [7]; this virus was also resistant to neutralization by hE16, whereas an SA58 variant encoding threonine at 332 [7] was efficiently neutralized (**Fig 1B**).

Two independent mouse challenge models were used to assess the protection provided by hE16 against the neutralization-sensitive and neutralization-resistant WNV strains and variants. Groups of outbred Swiss Webster mice, which are highly susceptible to peripheral challenge with neuroinvasive WNV strains, were given 100 μg doses of hE16 or phosphate-buffered saline (PBS) only and challenged 24 hours later with 10^2 plaque forming units (PFU) of each WNV strain/variant (equivalent to ~100 LD50s in each case). Alternatively, groups of inbred C57BL/6 mice, which are more resistant to WNV NY99 and have been used in previous evaluations of hE16 [3, 10] were challenged with 10^2 PFU of each virus and treated at two days after infection with 100 μg

hE16 or PBS only. The hE16 MAb afforded significant protection (90-100% survival) for mice in either the pre- or postexposure model against NY99 and variants that were efficiently neutralized *in vitro* by hE16 (TR330I and T332A/M). However, little or no protection was observed after challenge with the NY99ic T332K mutant or SA58 (**Table 1**).

Although mutagenesis of an infectious clone or *in vitro* selection can readily generate viruses resistant to hE16 and other MAbs, we questioned whether this commonly occurred under selective pressure during the course of treatment. To assess the potential for selection of resistant variants *in vivo* during MAb treatment, Swiss Webster mice pre-treated with 100 μg hE16 were exposed to a high dose (10^4 PFU) of NY99 virus. This challenge dose was chosen based on the known frequency of MAb escape variants selected from this virus population during earlier experiments [7] and because this represents a virus dose reliably introduced during feeding by *Culex.* mosquitoes [11]. All untreated control animals died by day 9 after infection (average survival time 7.4±1.0 days). Two of 10 treated mice showed signs of neuroinvasive disease and were euthanized on days 8 and 9. Virus was isolated from the brain of each mouse, and the isolates were found to encode single-nucleotide changes in E coding for mutation K307E and T332M. Both isolates retained the highly neuroinvasive phenotype of the parental NY99 virus (data not shown).

Immune dysfunction is a significant risk factor for development of severe WNV disease [reviewed in 12], suggesting that therapeutic antibodies and other treatments may be especially important for immunodeficient patients. To assess the potential for emergence of resistant variants during treatment of an immunocompromised host, a

group of 30 B and T cell-deficient *RAG* mice was exposed to a low dose (10^2 PFU) of strain WNV-NY2000 (3000-0259), which has an E gene sequence identical to that of NY99 [13], and then treated with 500 µg doses of hE16 administered one day after infection and at 14 day intervals thereafter. Although 24 mice remained healthy over a period of several months, six mice became ill and were either found dead or euthanized on days 18 (2 mice), 20 (2 mice), 32 (1 mouse) and 34 (1 mouse). Brains recovered from the latter two mice each yielded WNV encoding the K307E E protein mutation.

Consistent with previous studies [3], the recovered K307E variants were resistant to *in vitro* neutralization (**Fig 1C**) or *in vivo* protection (**Table 1**) by hE16, whereas the recovered T332M variant was only modestly resistant to E16 neutralization *in vitro* (data not shown) or *in vivo* (**Table 1**), similar to the NY99ic T332M variant.

DISCUSSION

The use of polyclonal or monoclonal immunotherapy is currently being explored for treatment of infections caused by many families of viruses and clearly offers a promising approach for postexposure treatment of flavivirus infections. In the case of hE16 for treatment of WNV infection, the presence of naturally occurring mutations in some WNV strains at the MAb binding site suggests that treatment coverage will not be universal, as evidenced by the resistance of lineage 2 strain SA58 *in vitro* and *in vivo*. Nonetheless, hE16 still afforded strong protection against variants encoding other mutations that increased resistance to neutralization but did not abolish binding. Analysis of more than 650 derived amino acid sequences in the NCBI Protein database representing human, avian, equine or mosquito North American WNV isolates during 1999-2005 identified none with amino acid variation at positions 307, 330, or 332, which could impair hE16 recognition and neutralization.

The present studies have also demonstrated the potential for *in vivo* selection of MAb escape variants. Although surprisingly infrequent, *in vivo* resistance may occur via selection of a pre-existing mutant subpopulation following exposure to higher doses of virus, a phenomenon which has also recently been reported in passively immunized monkeys exposed to 10^6 PFU doses of dengue virus type 2 [14]. Alternatively, resistance can develop through the emergence of variant viruses during prolonged treatment.

The length of time required for the emergence of the resistant K307E variants following 10^2 PFU challenge in *RAG* mice (up to 34 days) compared with the rapid selection of variants from the 10^4 PFU challenge in Swiss Webster mice (8-9 days), was somewhat surprising. Based on the inherent error rate of the flavivirus RNA polymerase

(~1 mutation per genome replication), the occurrence of resistant mutations at the genetic level should be expected to occur rapidly. The delay in emergence of resistance in *RAG* mice could be explained by packaging of early variant genomes in virions that also contain levels of wild-type E protein sufficient to allow neutralization by hE16. This is possible because flavivirus RNA can be encapsidated by structural proteins *in trans* [15] and complete neutralization (>99%) of WNV virions by hE16 requires occupancy of less than 50% of the available epitopes [6]. Although further studies are necessary, high levels of circulating hE16 with a long half-life may adequately neutralize virions comprised of wild type and resistant E protein variants to prevent rapid or eventual emergence of pathogenic escape variants, even in a highly immunocompromised subject.

Overall, these findings suggest that resistance to neutralizing anti-WNV antibody therapy can occur in vivo, although at frequencies that are perhaps lower than would be anticipated based on analogous studies of resistance to antiviral drugs that target virus replication. However, our data suggest that clinical evaluation of hE16 and other MAb therapeutics for flavivirus infections should monitor for selection or emergence of resistant variants, especially in immunocompromised patients, who are a likely target population for treatment of severe WNV infections. Such studies should include characterization of viral populations for detection of resistance and possibly the use in some patients of combinations of neutralizing antibodies that recognize distinct epitopes.

TABLE 1. Outcomes of infections of mice with West Nile virus strains or variants after pre- or post-exposure treatment with the neutralizing MAb hE16.

Mouse strain	hE16 Treatment*	Virus	hE16 treated		PBS only		Survival P value‡
			% survivors	AST±sd†	% survivors	AST±sd	
Swiss Webster	100µg day -1	NY99 wt	100	n/a	20	8.4±1.2	0.0003
		NY99ic T332A	100	n/a	10	8.1±0.3	<0.0001
		NY99ic T332M	90	8.0±0.0	0	8.4±0.8	<0.0001
		NY99ic T332K	30	7.4±0.8	10	8.8±1.5	0.8984
		NY99ic T330I	90	8.0±0.0	0	7.8±0.4	0.0008
		SA58	0	7.2±0.4	0	7.5±0.5	0.1704
		SA58 K332T	100	n/a	0	7.5±0.7	<0.0001
C57BL/6	100µg day +2	NY99ic wt	100	n/a	30	11.0±1.7	<0.0001
		NY99ic T332A	100	n/a	20	9.3±1.9	0.0003
		NY99ic T332M	100	n/a	30	10.7±1.6	<0.0001
		NY99ic T330I	100	n/a	10	9.4±1.8	<0.0001
		SA58	10	10.8±1.6	0	9.3±1.1	0.0207
		SA58 K332T	100	n/a	10	10.2±1.4	<0.0001
Swiss Webster	100µg day -1	NY99 K307E	10	8.8±1.3	0	7.8±0.4	0.0773
		NY99 T332M	70	13.3±3.8	0	8.0±0.7	0.0001

* Swiss Webster mice were treated with 100µg hE16 or PBS only, then challenged 1 day later with 100 PFU of indicated WNV strains (1000 pfu for NY99ic T332K). C57BL/6 mice were inoculated with 100 PFU of indicated WNV strains, then treated 2 days later with 100 µg hE16 or with PBS only. n=10 for each virus/treatment group, except for NY99 K307E/T332M "PBS only" groups, where n=5.

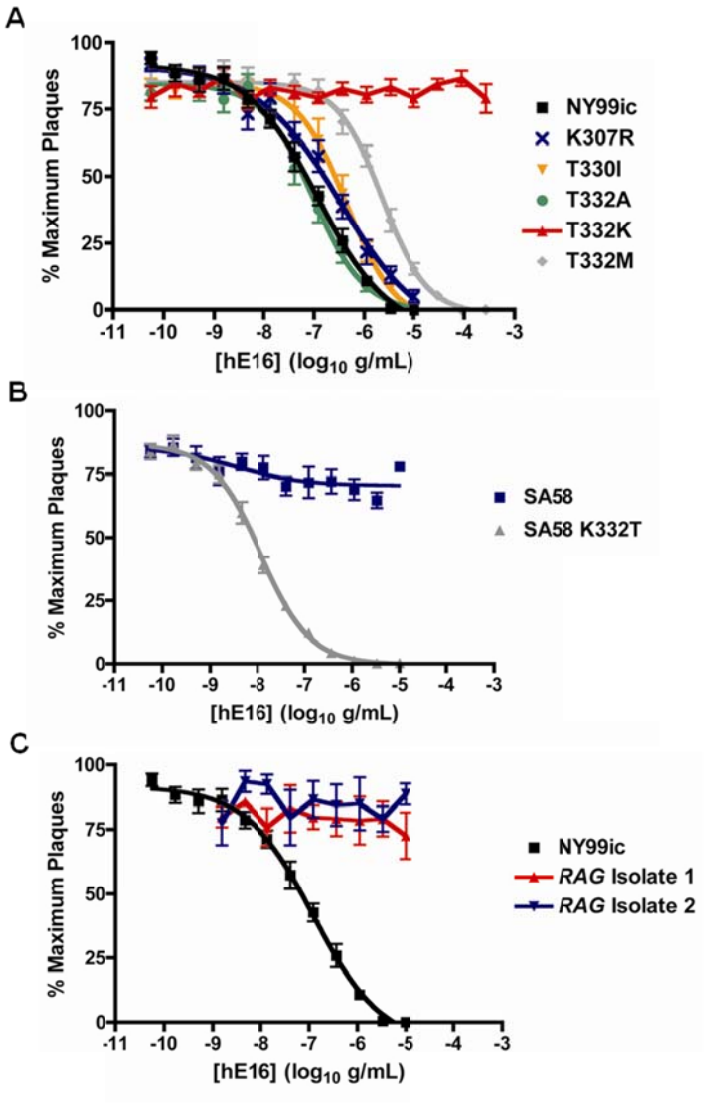
† AST – average survival time; sd – standard deviation; n/a – not applicable

‡ Significant differences in survival curves for hE16 treated vs. PBS only groups determined by log rank test using the Prism application (Graphpad Software).

FIGURE LEGEND

Figure 1. Neutralization by MAb hE16 of: (A) lineage 1 WNV strain NY99ic and K307R, T330I and T332A/K/M variants; (B) lineage 2 WNV strain SA58 and K332T variant; and (C) K307E escape variants selected *in vivo* from hE16-treated *RAG* mice. The data are an average of two to four independent experiments performed in triplicate on (A, B) Vero or (C) BHK21-15 cells.

FIGURE 1.


A
100
75
50
25
0
% Maximum Plaques
-11 -10 -9 -8 -7 -6 -5 -4 -3
[hE16] (log10 g/mL)
NY99ic
K307R
T330I
T332A
T332K
T332M
B
100
75
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0
% Maximum Plaques
-11 -10 -9 -8 -7 -6 -5 -4 -3
[hE16] (log10 g/mL)
SA58
SA58 K332T
C
100
75
50
25
0
% Maximum Plaques
-11 -10 -9 -8 -7 -6 -5 -4 -3
[hE16] (log10 g/mL)
NY99ic
RAG Isolate 1
RAG Isolate 2

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Curriculum Vitae

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Education

8/05-5/13 **M.D.**, Washington University School of Medicine, Saint Louis, MO

8/05-5/13 **Ph.D.** in Immunology, Washington University School of Medicine, Saint Louis, MO

8/01-5/05 **B.A.** in Biology, Washington University in St. Louis, Saint Louis, MO

Laboratory Experience

7/07-9/11 **Graduate Student in Laboratory of Michael S. Diamond, MD, PhD**
Studied the neutralization mechanism of West Nile Virus infection by human monoclonal antibodies and the mechanism of protection from infection in vivo by poorly neutralizing antibodies

6/04-1/05 **Research Assistant for Tamara L. Doering, MD, PhD**
Utilized an RNA interference genomic library screen to identify genes in Cryptococcus neoformans that affect overall viability and synthesis of the capsule, its main virulence factor

8/02-1/04 **Research Assistant for William R. Wikoff, PhD**
Continued efforts at crystallizing bacteriophage lambda terminase enzyme gpA subunit
Developed a successful crystallization screen for virus capsids, procapsids, and other large proteins

6/02-8/02 **Howard Hughes Medical Institute Undergraduate Summer Research Fellowship**
Worked to improve the solubility of the gpA subunit of terminase, the DNA packaging enzyme in bacteriophage lambda, for crystallization and subsequent x-ray crystallography
Laboratory of William R. Wikoff, PhD

Awards

4/11 **American Physician Scientist Association Poster Award**
One of six "Outstanding Poster Session Presentations" of 183 posters at ASCI/AAP Joint Meeting

4/11 **ASCI/AAP Joint Meeting Travel Award**
Received based on quality of abstract to help defray travel and lodging expenses

10/10 **Keystone Symposia Scholarship**
Received based on quality of abstract to help defray travel and lodging expenses

3/10 **Keystone Symposia Scholarship**
Received based on quality of abstract to help defray travel and lodging expenses

11/07 **American College of Physicians Missouri Chapter Physical Diagnosis Award**

Awarded to one student from each Missouri medical school based on performance in an outstanding fashion during the second year course in physical diagnosis

4/03 **Florence J. Moog Scholarship**
Awarded annually to a sophomore in Washington University College of Arts and Sciences who is preparing for a career in science based upon interest, achievement, and academic performance
Half –tuition for both junior and senior year

9/02 **PEW Midstates Science and Mathematics Consortium Travel Award**
Received based on quality of research to present results at an undergraduate scientific symposium at the University of Chicago

9/02 **Howard Hughes Medical Institute Travel Award**
Awarded to top two fellows of the 2002 HHMI Undergraduate Summer Research Fellowship
Used to attend the XVIII Biennial Conference on Phage/Virus Assembly at the Woods Hole Oceanographic Institute (6/03)

6/02-8/02 **Howard Hughes Medical Institute Undergraduate Summer Research Fellowship**
Awarded to 35 undergraduate students of Washington University in St. Louis

Presentations

Vogt, M. R., M. Engle, and M.S. Diamond (2011). Non-neutralizing antibodies protect against lethal West Nile virus infection via Fc-gamma receptor and complement–dependent mechanisms. The 3rd International Young Researcher Seminar in Zoonosis Control. Hokkaido University. Sapporo, Japan. Invited speaker.

Vogt, M. R., M. Engle, and M.S. Diamond (2011). Non-neutralizing antibodies protect against lethal West Nile virus infection via Fc-gamma receptor and complement–dependent mechanisms. ASCI/AAP Joint Meeting. Chicago, IL. Poster.

Vogt, M. R., M. Engle, and M.S. Diamond (2010). Non-neutralizing antibodies protect against lethal West Nile virus infection via Fc-gamma receptor and complement–dependent mechanisms. Keystone Symposium on Immunological Mechanisms of Vaccination. Seattle, WA. Poster.

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Teaching Experience

2009 **Immunology**
At request of the coursemaster, prepared concise notes highlighting the most important concepts of selected lectures. These notes were included in the course book distributed to all students of this first year medical school course.

9/07-3/08 **Cell and Organ Systems Biology – Histology**
Teaching assistant – led laboratory sessions and prepared exam reviews for first year medical students